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Faults and Fallacies in Microbiology

The Fourth Marjory Stephenson Memorial Lecture

BY G. S. WILSON

Public Health Laboratory Service, Medical Research Council, London

I need hardly say how much I appreciate the honour of being asked to give the fourth lecture in memory of Marjory Stephenson and how little I feel able to do justice to the task. Though many of you here knew her better than I did, I may perhaps be allowed to claim a small share in the success which she achieved. In the early twenties, when she was brooding over problems of growth and nutrition, she realized the necessity of using a quantitative technique. For this reason she came to Manchester where Topley had recently inaugurated the Diploma in Bacteriology course, and it was there that I had the privilege of teaching her how to count bacteria.

The choice of a subject for this lecture proved very difficult. As I have done no bench work for 14 years, it had to be a general one. I have always been interested in technique, and I therefore thought it might be worth studying the part played by faulty technique in reaching erroneous conclusions. I found an almost embarrassing richness of material before me, and I have perforce limited myself to a few illustrative examples.

Many of you will regard studying the errors of others as a presumptuous and invidious undertaking. I admit this. My purpose, however, is not, as that of some of the literary critics has been, to denigrate the outstanding men of the past, but to try and learn how lesser men can avoid falling into their errors. Even the greatest of scientists may go wrong, and all of us should realize the abyss at our side and say with John Bradford 'But for the grace of God there go I'. So far from attempting to act as a judge, I propose to be no more than an honest seeker after truth and of the ways of reaching it. I approach my subject, as every scientific investigator should, wearing 'the napless vesture of humility'.

Faulty technique

I shall start by quoting one example of the use of faulty technique which, though the best available at the time, has resulted in the perpetuation of error. In 1914 Brown, in India, made a careful study of the relation between the dry and moist weight of organisms, their numbers and their opacity. He was particularly impressed by the usefulness of opacity as a measure of the bacterial content of a suspension, and together with Kirwan (Brown & Kirwan, 1915) he introduced the following year a series of tubes labelled 1 to 10 made up with known amounts of a suspension of barium sulphate. These were very similar to those prepared by McFarland (see Kolmer & Boerner, 1941) in the United States of America. In 1924 Cunningham & Timothy published a series of tables purporting to show the number of organisms of several different bacterial species corresponding to each of Brown & Kirwan's opacity tubes. This would have been a useful table if only the bacterial counts had been made by a reliable method. Unfortunately the method used by Cunningham & Timothy was to stain the organisms with carbol-fuchsin and to count them on a haemocytometer slide with a high-power objective. Since the average diameter of the organisms they were counting was less than 1 μ, since the depth of a Thoma–Zeiss chamber is 100 μ, and since the staining of organisms from a 24 hr. culture is very irregular, it is not difficult to understand why this method underestimated the total number of organisms alive and dead in any given suspension. When a more accurate method is used, namely that of the Helber slide examined under dark-ground illumination with a low-power objective and a compensating eyepiece (Wilson, 1922), it is found that the figures for *Bacterium coli*, for example, in Cunningham & Timothy's table are only about one-third of the real number. The result has been that many of the figures quoted in bacteriological literature during the past 30 years are unreliable. The mistake is aggravated by the fact that opacity is a measure of the total bacterial content of a suspension and not of the number of living organisms in it, so that when these figures are used for calculating the number of organisms in virulence experiments they are often grossly misleading (see Wilson, 1926). Moreover, the figures corresponding to McFarland's opacity scale used by the Americans do not agree with those corresponding to the Brown & Kirwan tubes used in this country. Because of this a great deal of confusion arose when some years ago we were trying to repeat the American observations on the prophylactic value of a pertussis vaccine, and found that the bacterial content of a given vaccine was estimated quite differently in the two countries. In fact, though our results were subject to criticism on the ground that we were using too small a dosage, comparison showed that the British vaccine contained at least three times as many bacteria as the American one (see McFarlan, Topley & Fisher, 1945).

I suggest that the World Health Organization might well consider the introduction of a standard method of counting which would do away with the present anomalies.

Failure to control one's technique

This is perhaps the commonest failure of all, and I could give you several examples of it. Let me start on the filtrable forms of the tubercle bacillus.

Filtrable forms of the tubercle bacillus

In 1910 Fontes, in Brazil, filtered some caseous pus through a Berkefeld candle and injected the filtrate subcutaneously into guinea-pigs. After a fortnight one of the animals showed a swelling of the inguinal glands; no acid-fast bacilli were visible microscopically. The spleen of this animal was inoculated into fresh guinea-pigs; when the animals were killed 5 months later acid-fast bacilli were found in the lymph nodes and lungs. These observations were confirmed by numerous workers. In general, it was observed that when pure cultures of tubercle bacilli or tuberculous products were ground up and filtered through a Berkefeld or Chamberland candle, and the filtrate was injected in 1–10 ml. quantities subcutaneously into guinea-pigs, a peculiar disease was liable to follow which differed widely from the usual picture of experimental tuberculosis. The animals survived indefinitely or died after 2–4 months. At necropsy there was no local lesion; one or more groups of lymph nodes—particularly the tracheobronchial—were swollen but rarely caseous; and at times small lesions were found in the spleen or lungs. Careful examination with the microscope revealed a few acid-fast bacilli in the lymph nodes or other lesions. When the nodes were injected into fresh guinea-pigs, the same type of disease followed. The conclusion was drawn that the tubercle bacillus passed through a developmental cycle, one stage of which was characterized by the presence of minute filtrable forms capable of giving rise to a modified form of tuberculosis in experimental animals. It was even supposed by Calmette that these filtrable forms were able to pass through the placenta and infect the foetus (Calmette, Valtis, Nègre & Boquet, 1925; Calmette, Valtis & Lacomme, 1926).

This kind of work is beset with technical errors, and unless these are avoided by suitably controlled observations all the way, they are liable to give rise, as here, to unjustified conclusions. Porous earthenware filters cannot be properly standardized and may occasionally let through the odd bacillus. The type of tuberculosis that follows the inoculation of guinea-pigs with minimal numbers of tubercle bacilli is one that progresses very slowly, causes little caseation in the lymphatic nodes, and is characterized by healing of the proximal lesions and gradual spread of the distal (Schwabacher & Wilson, 1937).

Acid-fast bacilli in the mediastinal and mesenteric lymph nodes are not uncommon in healthy guinea-pigs as the result apparently of their inhalation or ingestion in hay and straw. Caseous material, and particularly broth cultures, may contain tuberculin which is responsible for the cachexia so often observed. Moreover, the type of tuberculin that was used in these experiments to follow the course of infection may contain acid-fast bacillary particles capable of causing confusion on microscopical examination. These are some of the errors involved, and it is noteworthy that those workers who took care to avoid them were unable to reproduce the lesions observed by Fontes and his followers. I do not wish to deny the existence of a filtrable stage of the tubercle bacillus, but I do wish to point out that the conclusion in favour of its supposed existence might never have been drawn if the technique used to support it had been properly controlled from the start.

Tuberculous bacillaemia

Another example of failure to take sufficient precautions against error is provided by the long series of observations, culminating in those of Löwenstein and his followers, on the occurrence of tuberculous bacillaemia. Pulmonary tuberculosis is one of the few diseases in which a laboratory diagnosis can be made with reasonable certainty by microscopical examination alone. For this reason the examination of sputum and of other material of pathological origin has often been left to persons with little or no bacteriological training, who were unaware of the numerous pitfalls that exist in this type of work. Tuberculosis has always been a hunting ground for the bacteriological tyro, and probably more errors have been promulgated in relation to this disease than to any other.

Many of the early workers reported the microscopical finding of tubercle bacilli in the blood of a high proportion of tuberculous patients and even of non-tuberculous persons and of patients suffering from other diseases. They did not appreciate the deceptive acid-fast artefacts that may be present in stained films of blood, particularly those derived from the lipid coat of the lysed red blood corpuscles. Nor did they take precautions against the contamination of their materials with the multiple saprophytic acid-fast bacilli that exist in dust, in reagents made up with old distilled water or with tap water, and in leather and rubber washers.

To confirm their claims, a number of these workers carried out animal inoculation. Ever since the time of Robert Koch this method has been recognized as the most delicate of all methods for demonstrating the presence of tubercle bacilli, so that positive results by guinea-pig inoculation are naturally regarded as being conclusive.

It came as a great shock to me, when some years ago I was called upon to investigate these claims, to find that many of those who had used this method had completely travestied its whole purpose. Instead of using it to show that the bacillus in question was able to set up a progressive, generalized, and fatal disease in the guinea-pig, characterized by the development of lesions typical in their appearance and distribution and containing acid-fast bacilli, they had contented themselves with diagnosing experimental tuberculosis by finding acid-fast bacilli in the spleen in the complete absence of macroscopic lesions or by relying entirely on a positive tuberculin reaction. The occurrence of natural tuberculosis and of other diseases simulating tuberculosis in some respects, caused by organisms of the *Pasteurella*, *Brucella* and *Salmonella* groups, provided an added stumbling block.

The extravagant claims made by some of the early workers on the frequency of tuberculous bacillaemia appeared to receive abundant confirmation by Löwenstein and his colleagues (for references see Wilson, 1933) at Vienna in the early thirties of this century. Löwenstein devised an excellent egg medium for the growth of tubercle bacilli, and by its means was able to report the isolation of these organisms from the blood of patients in the early stages of both pulmonary and non-pulmonary tuberculosis. Passing on to other diseases, Löwenstein and his followers claimed success in demonstrating the presence of tubercle bacilli in the blood of patients suffering from various forms of skin disease, from polyarthritis, from chorea, rheumatism, multiple sclerosis, retrobulbar neuritis, schizophrenia, polycythaemia rubra, endocarditis and tenosynovitis.

What was the explanation of results that common sense alone would consider ridiculous? There were many reasons which I have no time to describe in full. Two of the principal ones were that: (*a*) cultures were considered positive when, in the absence of visible colony formation, smears from the surface of the medium showed acid-fast bacilli on microscopical examination; and (*b*) smears were often made on slides which had been used previously for tuberculous specimens stained by Ziehl–Neelsen. Some of the macroscopic cultures that were obtained were almost certainly cultures of saprophytic acid-fast bacilli from the environment and not of true tubercle bacilli.

Critical examination of the results obtained by workers who exacted a high standard of technique and interpretation shows that tuberculous bacillaemia is seldom demonstrable during life, except in generalized tuberculosis or during an advanced stage of pulmonary tuberculosis. To reach this conclusion, however, and to refute the claims of Löwenstein and his associates to have provided a method for the early diagnosis of tuberculosis necessitated the devotion of a large amount of work by over a hundred observers in different parts of the world. The claim was of such tremendous clinical importance that it could not be neglected, and in consequence a great deal of time that might have been devoted to a better cause was wasted.

It fell to me to draw up a critical appreciation of this work for the Medical Research Council, and I shall never forget the late Sir Walter Fletcher's remark when I went down to Old Queen Street to discuss the report with him personally. He said: 'You know, Wilson, I am a physiologist, and I know that unless a physiologist uses a good technique he gets no results; but it seems to me that in bacteriology the worse your technique is, the more astounding are your results.'

Supposed cultivation of viruses from poliomyelitis, influenza, and numerous other diseases

One of the most extraordinary chapters in the history of bacteriology concerns the supposed isolation of filtrable organisms from a variety of diseases by means of the ascitic fluid kidney culture technique. It is all the more extraordinary in that some of the foremost bacteriologists in the world, such as Simon Flexner and Hideyo Noguchi, were among those who fell into error.

In 1911 Noguchi introduced a medium for the cultivation of spirochaetes. It was a modification of Schereschewsky's (1909) medium and consisted essentially of 15–20 ml. of sterile unfiltered serum or ascitic fluid contained in a tall test-tube. On the recommendation of Theobald Smith a small piece of freshly excised sterile rabbit kidney was added to each tube. The medium was covered with a layer of liquid paraffin 1–2 cm. deep to shut out the air. Sometimes agar was included. Noguchi had considerable success with this medium and, though few workers now would be prepared to agree with his claim to have cultivated virulent *Treponema pallidum* (1911), he did succeed in cultivating a number of other spirochaetes (Noguchi, 1912*a*, *b*, *c*). This medium apparently owed its virtue to the natural animal protein provided by the serum or ascitic fluid and to the anaerobic conditions prevailing around the fragment of kidney.

Encouraged by his success, Noguchi (1913) turned his attention to other organisms that had not yet been cultivated. The first one he chose was the parasite of rabies. He inoculated his medium with a fragment from the central nervous system of a rabid animal, and described how during the following days an opalescent haze appeared around the tissue. Microscopical examination revealed the presence of what appeared to be pleomorphic, often nucleated, particles, staining with Giemsa, and ranging in size from the merest granules to multinucleated bodies 12μ in diameter. Many of these bodies bore a close resemblance to Negri corpuscles, as was evident from photomicrographs provided for comparison. Noguchi was able to transfer the supposed parasite to fresh tubes of medium and, by inoculating cultures containing the granular, pleomorphic or nucleated bodies into animals, to reproduce the disease in dogs, rabbits and guinea-pigs. Film preparations from the brain of the animals contained the granular and sometimes the nucleated bodies in large numbers. Noguchi regarded the organism as probably a protozoon.

Closely following this work came a description by Flexner & Noguchi (1913) of the cultivation of the organism of poliomyelitis. The same technique was used, and the same type of turbidity developed around the tissues. Sometimes culture was successful when the inoculum consisted of a suspension of brain filtered through a Berkefeld V candle instead of a fragment of tissue. When agar was incorporated in the medium minute colonies could be seen extending up to within 2 cm. or so of the surface. Microscopical examination revealed the presence of minute globoid bodies, 0·15–0·3μ in diameter, arranged in pairs, short chains, and small masses, staining by Giemsa and tending to be Gram-negative when young and Gram-positive when old. Several experiments were performed in which monkeys are said to have developed poliomyelitis after intracerebral or intrasciatic injection with third- or fifth-generation cultures as well as with brain from passage monkeys. Not all the inoculated animals developed the disease, but in those which did typical histological findings were reported in the central nervous system. Moreover, by a special staining technique globoid bodies were found in smears and sections of the nervous tissue.

The article in which this work is reported is a long one; the monkey inoculations are described individually; numerous photographs and photomicrographs are provided; and it is written by experienced workers from the Rockefeller Institute who were aware of most of the pitfalls. Apart from the absence of certain desirable controls, the article could hardly help but carry conviction to the unbiased reader.

After the apparent success with this medium in the isolation of the causative agents of rabies and poliomyelitis, Foster (1917), a major in the Medical Corps of the United States Army, described the cultivation of a filtrable virus from the common cold. Both the macroscopic and the microscopic appearances in the cultures were very similar to those reported by Flexner & Noguchi for poliomyelitis, but the globoid bodies were more variable in size and the larger ones among them showed signs of budding. Subculture into ascitic fluid containing agar resulted in the development of minute colonies around the kidney at the bottom of the tube. Second-generation cultures in fluid medium filtered through a Berkefeld N candle and instilled into the nose gave rise to colds in 11 out of 11 human volunteers, and from 7 of them the virus was recovered in culture. Much the same findings were recorded in the same year by Dold (1917) working at Shanghai, though Dold was unable to see anything distinctive in the microscopical picture.

It is not surprising that, at a time when the findings of Flexner & Noguchi were credited with the full weight of authority that lay behind them, workers should look for other diseases to which to apply the new method.

At the 20th General Hospital of the British Expeditionary Force in the area of Étaples, Bradford, Bashford & J. A. Wilson (1918–19*a*), after an apparent preliminary success in isolating the aetiological agent of acute infective polyneuritis, turned their attention to the great problem of influenza. They investigated cases of the disease during the severe outbreaks in the autumn of 1918 and the early spring of 1919 (1918–19*b*). Working in the strictest secrecy, surrounded by an armed guard, and out of contact with all colleagues, J. A. Wilson recorded the isolation of the influenza virus in Noguchi medium from 22 out of 25 samples of blood, 14 out of 16 pleural fluids, 40 out of 40 sputa, as well as from odd specimens of bile, urine, and cerebrospinal fluid, while Bashford reproduced the disease with cultures in guinea-pigs, rabbits and monkeys. Subcutaneous injection was usually without effect, but intravenous and subdural inoculations were almost invariably followed by the disease. The virus was recovered from the inoculated animals.

Not content with this achievement, Bradford, Bashford & Wilson (1919) applied the same technique to other diseases, and were soon able to record the presence of filter-passing organisms in material from cases of trench fever, acute nephritis, measles, rubella, mumps, typhus and encephalitis lethargica. The latter part of this work was transferred from Étaples to the Lister Institute, and was regarded as so important that demobilization of the laboratory assistants was voluntarily delayed in order to allow of its completion.

It was time for the bubble to be pricked. Already Kraus & Barbará (1914) in Germany, attempting to repeat Noguchi's findings in rabies, had been able to confirm the presence of pleomorphic chromatic corpuscles and granules in cultures, but had pointed out that apparently identical bodies were present in normal ascitic fluid. Moreover, they had failed to reproduce the disease in animals by means of cultures. Probably because of the war this important paper was overlooked, and it was left to Arkwright (1919) to bring the whole unhappy tale to an end. Examining Wilson's cultures, he found that many of them were contaminated with staphylococci and diphtheroid bacilli. These organisms often stained only in the centre or at the poles and consequently appeared like minute globoid bodies. In cultures that were sterile he observed the presence of smaller or larger granules, $0{\cdot}2$–$0{\cdot}3\mu$ in diameter, globoid in shape or bipolar-stained, and showed that these consisted not of viruses but of particles of protein precipitated by the acid resulting from autolysis of the piece of kidney at the bottom of the tube. Discussing these results, Arkwright attributed the mistakes that had been made to working in isolation, to failure to exclude contamination by subculture on to ordinary media, to undue reliance on the efficacy of porcelain filters for removing bacteria, and to inadequate staining. It is only fair to add that at the end of Arkwright's paper Bradford and Wilson withdrew their claims.

From a careful study of the papers in which work with Noguchi's medium is reported, I find it hard to explain many of the results, particularly those following the inoculation of cultures into animals. The difficulty of interpreting the appearances in stained films from fluid cultures in which the protein is precipitated by heating is understandable, and all the more so when the medium contains blood or serum and has been incubated for 5 days or more till a deposit has formed. That a variety of pleomorphic bodies should be seen under such conditions is not surprising, but it is rather surprising that they were said to have been absent from control cultures.

The risk of contamination in putting up Noguchi tubes hardly needs emphasizing. Each tube has to be opened at least five times before the culture is ready for incubation. Arkwright, who put up a series of tubes himself using material from trench fever, found that 20 % were contaminated—a proportion substantially the same as that of control tubes, namely 22 %.

The technique of filtration used by Wilson was crude. He obtained his negative pressure by attaching his Berkefeld or Massen candles to a cooling autoclave and filtered for 1–4 hr. It is not to be wondered at that in these circumstances some micro-organisms were sucked through.

The pathological appearances produced by the intravenous or subdural injection of cultures into animals and believed by Bashford to be characteristic of influenza may at times have been due to staphylococci, particularly as nephritis was often recorded on post-mortem examination. Dark patches and haemorrhages are not uncommon in the lungs of guinea-pigs killed by various means in the laboratory, and had more attention been concentrated on injections with control cultures the pathological appearances noted might have been interpreted more correctly.

In their work on poliomyelitis Flexner & Noguchi put up control tubes consisting of kidney and ascitic fluid, or of infected brain and ascitic fluid, but not tubes containing ascitic fluid, kidney and normal brain. Similarly, in their experimental work on animals, they did not inject cultures made with normal brain tissue. Nevertheless, it is not easy to understand why Flexner in particular should have gone wrong in claiming to have reproduced poliomyelitis by the injection of cultures containing globoid bodies. Working with Lewis in 1910, Flexner had reproduced the disease in monkeys with human material, and had carried it over from monkey to monkey by subdural injection of cord suspensions. He had had, therefore, a considerable experience of the experimental disease, and yet in his work with Noguchi he must have been mistaken. The diagnosis, however, of experimental poliomyelitis in monkeys is by no means straightforward, and here again it is clear from Sabin's (1959) recent article that the technique used is all-important.

It is well to realize that probably most of the erroneous conclusions based on the use of Noguchi's medium would have been avoided if only a full set, instead of a partial set, of controls had been systematically used throughout the investigations.

The danger of working in isolation referred to by Arkwright needs stressing. It is not only complete isolation that should be avoided, but relative isolation as well. Lone workers in the provinces, the colonies, and sparsely populated lands are not exposed to that healthy criticism which most workers in large towns enjoy. If there is a fault in their technique, there is no one to point it out to them. If their erroneous conclusions or ideas are not challenged at the start, they are liable to become fixed. When eventually they are challenged they have become so deeply implanted that their attempted refutation is often regarded as a personal insult, and is accompanied by an emotional reaction that is quite foreign to workers who are used to free discussion with their colleagues. Though, as we have seen, big institutes are not immune from error, research carried out in isolation is especially perilous, and no one who is wise will publish a discovery of any moment without seeking the opinion of one or two trusted experts in the field.

Confusion of natural and experimental disease

Confusion may be caused not only by the lesions of a natural disease but by latent infection in apparently healthy animals. This was responsible for some of the statements in the early German literature on food poisoning that carcass meat which appeared to be perfectly good on inspection nevertheless often contained organisms of the paratyphoid–Gärtner group. Mühlens, Dahm & Fürst (1909), for example, fed white mice on uncooked, pickled, and smoked meat that was apparently sound and found that over 50 % of the animals died. From a considerable proportion of the dead mice Gärtner's bacillus was isolated. Direct culture tests on the meat were negative. This led the authors to conclude that the organisms must have been present in the meat but in only very small numbers.

It was not long before other workers in Germany pointed out the fallacy of this conclusion. Mice are subject to natural infection with Gärtner's bacillus, and the injection of foreign material may stimulate a latent infection into activity. The same fallacy underlay Nocard's (1893; see 1897) conclusion that psittacosis was caused by *Salmonella typhimurium* and M'Gowan's (1911) and Ferry's (1911) conclusion that dog distemper was caused by *Haemophilus bronchisepticus*.

It is sometimes difficult to know for certain whether an organism isolated from an animal was present in the injected material or was derived from the animal's own tissues. Errors due to this cause can generally be avoided when the organism in question is known to be a potential parasite of the animal that is under test. Difficulty arises, however, when the animal inoculation method is used to search for an organism whose identity is unknown. The virologists have been particularly troubled in this type of work. Francis & Magill (1938), for example, isolated a meningo-pneumonitis virus from ferrets that had been inoculated with throat washings of human patients suffering from an epidemic influenza-like disease. Whether the virus was derived from the patients or from the ferrets it was, however, impossible to say. The difficulty is increased by the tendency of some animal parasites that cause latent infections to be practically avirulent and hence almost impossible to demonstrate. This is true of the mouse pneumonitis virus described by Horsfall & Hahn (1940). Under ordinary conditions it appears to be avirulent, but by serial passage through mice inoculated intranasally with infective lung it becomes capable of causing a disease characterized by pulmonary consolidation. The encephalomyelitis virus of mice described by Theiler (1937) is another virus that is sometimes so widely spread in mouse colonies that by the time the animals are a month old practically every one of them has been infected. The infection remains latent throughout life, but may be stirred up by the injection of foreign material. It is specially liable to cause confusion to workers looking for neurotropic viruses.

In this connexion it is interesting to note that the existence of a latent infection with one organism may enhance the virulence of a different organism. Gledhill & Niven (1957), for example, found that mice infected with *Eperythrozoön coccoides* were more sensitive than normal mice to the injection of filtered or formolized cultures of *Salmonella typhimurium*. This observation was of peculiar interest to me, because it made me wonder whether it might have a bearing on the results obtained by Topley and his co-workers in their studies on the epidemiology of mouse typhoid. In some of my own work, which I never published, I observed striking differences from month to month in the mortality of mice injected with the same dose of the same strain of *S. typhimurium*. Similar differences, though not necessarily concurrent, were observed in mice injected with a lipopolysaccharide fraction extracted from the strain at the beginning of the work. The mice were in groups of 30, and were injected once a month over a period of 5 years. As far as possible all normal variables were controlled. The differences observed were not seasonal in the ordinary sense, because periods of high mortality might occur at any time of the year, nor could any regularity be discerned in the curves formed by plotting the death rates. Neither Topley nor I at the time had considered the possibility of a latent bartonella or eperythrozoal infection. The mice were bought from different dealers and, though free from salmonella infection, may quite easily have been infected with one of these blood parasites. It is a matter for speculation to what extent such an infection may have been responsible for the irregular results obtained. Certainly in any future work on the epidemiology of a disease such as mouse typhoid care would have to be taken to exclude as far as possible the presence of latent infections with other organisms.

Confusion may occur not only between natural and experimental disease in animals, but between natural diseases occurring in a given species of animal or in man. A good example of this was the failure of the clinicians to distinguish between yellow fever and Weil's disease that led Noguchi to identify *Leptospira icteroides* as the cause of yellow fever (see Noguchi, 1925). In dogs the difficulty in differential diagnosis between the encephalitic manifestations of distemper, of hard pad disease, and of so-called hysteria has set laboratory workers a problem of great complexity in deciding whether one virus is responsible or whether two or more are concerned.

Generally speaking, it is advisable in the investigation of any disease to define it clinically and epidemiologically before serious work on it is commenced in the laboratory. This may be a counsel of perfection, and admittedly bacteriological or virological examination may be necessary to reveal the difference between two clinically similar diseases of different aetiology. But failure in the past has at times resulted from neglect to study the epidemiological behaviour of a given disease with the same care as its clinical manifestations. It took, for instance, many years of epidemiological study to show that infectious hepatitis and serum hepatitis, though clinically alike, nevertheless behaved in their incidence, their mode of spread, and their infectivity as separate diseases. Once this was realized, and once it was appreciated that arsenic jaundice, homologous serum jaundice, and transfusion jaundice were all different expressions of one and the same disease, namely serum hepatitis, the interpretation of the laboratory findings, though still not definitive, was made very much easier.

Failure to appreciate the complexity of the situation

Though this hardly comes within the definition of technique, it is a mistake that has been frequently made by both medical and non-medical microbiologists. It is appropriate to refer to it to-day because, as Woods (1953) pointed out in his inaugural lecture, it was Marjory Stephenson, among others, who realized the danger of drawing conclusions from the complex picture generally presented by Nature, and who indicated the ways in which progressive simplification might be effected in order to render the role of individual organisms more amenable to study. It would be almost equally apposite to stress the opposite danger, namely that of generalizing on the basis of observations made in too simple an environment. Biology can progress only by using both inductive and deductive reasoning, and it is when one of these is used to the exclusion of the other that false conclusions are likely to be reached.

One of the earliest examples is Metchnikoff's hypothesis of intoxication resulting from the absorption of poisonous products generated by the proteolytic flora of the human intestine. Under the influence of Pasteur the fermentative activity of the intestinal bacteria had been regarded as assisting in digestion of the food taken in by the host. Metchnikoff (see Metchnikoff, 1921; Schieblich, 1929), on the other hand, who was concerned with the question of longevity, came to regard the products of putrefaction as responsible for the degenerative processes in the heart, arteries, and other essential structures of the body that led to a premature end of human existence. To combat this effect he proposed to replace the proteolytic organisms of the intestine by a lactobacillary flora. This he did by the ingestion of fermented milk, such as the Bulgarian yoghurt, containing organisms that broke down the lactose to lactic and other fatty acids. The fact that Metchnikoff himself died at the age of 71 is hardly a sufficient proof of the fallacy of his thesis, but subsequent experience on a wider scale has failed to substantiate it in any way. It has proved impossible to establish a lactobacillary flora in the adult human gut without the ingestion of impossibly large quantities of fermented milk, or of lactose or dextrin. Moreover in animals, and probably also in man, it has been found that the natural intestinal bacteria lead to the synthesis of amino acids and of vitamins that play an important part in the nutrition of the host. As the recent work on the growth-promoting powers of chlortetracycline in farm animals has shown, the subject is, however, very complex, and the problems presented will take many years of study to resolve.

Other examples that might be quoted of over-simplification are the attempt to reproduce the defence system of the human body by mixtures of blood and organisms in a capillary tube, the danger of arguing by analogy from animals to man, of which I could quote many instances, and the evaluation of a disinfectant in practice by its performance under very simple and artificial conditions in the laboratory. The hope, too, that the cholera bacteriophage would behave in the complex environment of the human intestine in the same way as it did in a pure culture of cholera vibrios in the test-tube may perhaps be regarded as a further instance of the danger of over-simplification. It is not, of course, that any of these attempts was wrong in itself. In making them the authors were merely doing what Marjory Stephenson would undoubtedly have approved of. It is because far-reaching conclusions were drawn from them not justified by the evidence that I refer to the danger of over-simplification.

One of the most fertile reactions against this danger was Sir Henry Dale's contention that the standardization of biological products could not be undertaken by the same methods as those used for chemical substances. It was this that led to the development of the present world-wide organization for the control of biological products, and helped also to introduce the statistical method of approach into so many of the problems of microbiology and immunology.

Topley likewise reacted against over-simplification. At the time he started his main work towards the close of the First World War the literature was full of statements on the virulence of organisms, and particularly on their change in virulence as the result of some natural or artificial procedure, which was based on experiments made on minimal numbers of animals. Topley soon realized that the reaction of a given animal to a particular challenge was determined by a number of different factors, and that to attempt to draw far-reaching conclusions without taking account of the natural variation among animals belonging to a given group was to present a grossly over-simplified and often erroneous picture of reality. By enlisting the co-operation of Greenwood he was able to study the limits of natural variation and to plan his experiments in such a way as to make reasonably sure by virtue of the number of animals used that the answer obtained would have at least a certain degree of probability.

The importation of the statistical method by Dale and his followers into the realm of biological standardization and by Topley and his followers into the realm of immunology has profoundly affected the whole outlook of clinical and laboratory medicine, and has made possible the numerous field trials and investigations in which this country has excelled of recent years. The statistical method provides a compromise between the over-simplified environment of the test-tube and the uncontrolled complexity of the natural environment. So successful has its application been that, unless its limitations are appreciated, it may lead to the substitution of quantity for quality. In spite of its success, its use is to some extent a confession of failure—failure to make the perfect observation; and it should not be forgotten that the value of the statistical method is in proportion to the quality of the observations to which it is applied. It should never be offered as an excuse for laziness.

Conclusions

There are many other faults and fallacies which I might describe if time permitted, such as the mistakes that have been made in wrongly identifying the cause of a disease by failure to conform to Koch's postulates; overemphasis on the numbers rather than the nature of the organisms in assessing the quality of food substances; the unjustifiable transference of a generalization that is true in one department of microbiology to another department in which the premisses on which the generalization rests do not hold, such as the significance of *Escherichia coli* as an indicator of human excretal pollution; and in the field of epidemiology the drawing of general conclusions for a whole country from observations based on only limited parts of it.

The few examples I have quoted may, however, suffice to show how closely fact and fancy are woven into the tissue of scientific investigation. The younger workers who begin where the older ones leave off do not realize how much error has been perpetrated in arriving at the corpus of truth with which they start. Nearly all the errors of which I have spoken have arisen from imperfect technique and absence of full controls, or from over-simplification through failure to appreciate the complexity of the situation.

The conclusion I reach is that the scientific investigator should receive a wide general training, including the historical background of his subject, together with particular instruction in experimental technique and the errors of different techniques. The late Lord Horder used to say that every medical student should study elementary logic. This is good advice, but I should prefer to replace elementary logic by elementary statistics. Logic is excellent in its own sphere, but when applied to biology it may lead to curious results. The statistical approach is a better one, because it takes account of the concept of variation.

Another conclusion I would draw is the desirability of implanting a healthy scepticism, though not disrespect, of authority. Great men often make great mistakes, and microbiologists are no exception. Conversely, it is probably true to say that no fallacy is ever propagated without the support of some great man behind it. Whenever objective evidence is obtainable it is our duty to study that rather than to accept uncritically the conclusions that have been drawn from it by others. When, of course, objectivity cannot be obtained, as in the measurement of beauty and goodness and in the realm of religion, then tradition, authority and our own personal experience must serve as our guide.

Many people, such for example as Beveridge (1950), have tried to define the qualities that make a good research worker. I have tried to do the opposite, and examine the reasons for the production of bad research work. You have listened patiently to me, patiently to what may well be considered a series of dull platitudes, and as I finish I expect you will heave a sigh of relief and say with Hamlet 'These tedious old fools'.

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Transduction of Streptomycin Resistance in *Salmonella typhimurium*

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SUMMARY: In contrast to the failure of several previous workers to transduce streptomycin resistance, it was found that streptomycin indifference (one-step complete resistance) could quite reproducibly be transduced. For the expression of indifference after transduction, the infected bacteria must be allowed to divide at least once. This finding is assumed to reflect a segregation delay because of the recessive nature of the indifference locus. But from the fact that transduced indifference is phenotypically expressed from partial to complete resistance, the participation of a phenomic lag is also assumed.

In addition to the delay of phenotypic expression, several other aspects of transduction of indifference were studied and a close similarity of the behaviour of indifference to other chromosomal markers was found.

The transduction of one-step intermediate resistance into sensitive and into other one-step intermediate resistant recipients, however, was found to be difficult to demonstrate if it occurs at all. The difficulty of detecting resistant transductants is apparently not due to the killing of resistant bacteria in the presence of sensitive cells.

The difficulty of transducing intermediate resistance is assumed to be due to a selective disadvantage of the resistant, slow-growing transductants. The failure to find transductions even with slow-growing sensitive recipients may have been due to the fact that successful transductions yielded even slower growing resistant clones.

On the other hand, the transduction of second-step resistance into one-step resistant mutants was found to take place and the transduction of indifference into one-step resistant bacteria yielded slow-growing indifferent transductants.

Cavalli (1952) discovered multiple loci for chloramphenicol and terramycin resistance by the use of recombination in *Escherichia coli*, strain K-12; on the other hand, Demerec (1950) and Newcombe & Nyholm (1950) reported that streptomycin resistance is controlled by a single locus or by very closely linked loci in the same bacterial strain. We have reported that in streptomycin resistance the complete resistance which is developed in a single step with low mutation rates is different in various aspects from the multiple-step resistance, and we have given the name of streptomycin indifference to one-step complete resistance (Watanabe, 1954; Watanabe, Fukasawa & Ushiba, 1957). The genetics of the apparently one-step complete resistance has been studied by many workers, but much attention has not been paid to multiple-step resistance, although Hotchkiss (1951) clarified the multiple-step nature of penicillin resistance and reported that streptomycin resistance is developed in both single and multiple steps as revealed by transformation studies in *Diplococcus pneumoniae* (Hotchkiss, 1952).

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Since transduction was discovered by Zinder & Lederberg (1952), and it was reported that streptomycin resistance is transducible, transduction has been thought to be an excellent technique for the study of the genetics of drug resistance. However, several workers (see Bryson & Szybalski, 1955; and Bryson & Demerec, 1955), including Zinder himself (personal communication), have been unable to reproduce their transduction experiments on resistance to streptomycin as well as to other drugs in *Salmonella typhimurium* strain LT-2 which was originally used by Zinder and Lederberg. There are some workers who reported that streptomycin resistance is transducible (in *Salmonella typhosa*, Baron, 1953; in *S. anatum*, Iseki & Sakai, 1954; in *S. typhimurium*, Shimizu, 1955; in *Escherichia coli*, Lennox, 1955), but it is not clear which type of streptomycin resistance is involved in their studies. We believe that it is important to identify the type of streptomycin resistance whenever we deal with it, because, as will be reported in this paper, streptomycin resistance involves multiple loci, and not only are their phenotypes apparently different from one another but it is also likely that the biochemical mechanisms of resistance controlled by these loci are different.

In the first part of this paper we will report on the transduction of streptomycin indifference, about which much data have been already accumulated by other genetic methods and which is obviously controlled by a single locus or by very closely linked loci. In the following part, studies on the transduction of multiple-step resistance into sensitive or resistant bacteria will be reported.

METHODS

Strains and media

Throughout the present studies, *Salmonella typhimurium*, strains LT-2 and LT-7, and temperate phage PLT-22 were used. Both of the bacterial strains were found to show rather high resistance to streptomycin on ordinary nutrient media and, because this high resistance has made the fine analysis of stepwise increase or decrease of streptomycin resistance difficult, it was decided to use low phosphate medium. It has already been established that phosphate reverses the action of streptomycin (Watanabe, 1954; Hurwitz & Rosano, 1958). The low phosphate medium had the following % composition: NH_4Cl 0·05, NH_4NO_3 0·01, Na_2SO_4 0·02, K_2HPO_4 0·03, KH_2PO_4 0·01, $MgSO_4$ 0·01, $CaCl_2$ 0·001, yeast extract (Difco) 0·5, NZ-case (Sheffield) 0·5, (agar 1·5).

Isolation of mutants

The streptomycin indifferent mutants were obtained by exposing a large number of wild-type bacteria to more than 500 μg. of streptomycin sulphate per ml. In lower concentrations of the antibiotic, intermediate resistant mutants were selected as well. After repeated single colony isolations, the indifferent bacteria were grown in liquid medium with aeration and during logarithmic growth they were infected with phage grown on wild-type bacteria with a multiplicity of input of about 10^{-3}. This low multiplicity was chosen in order to let all the phage particles multiply on the mutant bacteria. The infected bacteria were further aerated at 37°, and after 5 hr. the culture was centrifuged and the supernatant treated with chloroform. Phage titrations were carried out by the method of Adams (1950).

For isolating intermediate streptomycin resistant mutants independently, overnight cultures in 1 ml. of liquid medium were obtained starting from about 100 cells in each culture. One-tenth ml. of each culture was plated on agar medium containing 25, 50, 100 and 250μg. streptomycin/ml. and after 5 days' incubation colonies were picked at random, re-isolated on to streptomycin-free agar plates, and used as first-step mutants, although some of them might be multiple-step mutants. When two-step mutants were required, first-step mutants were plated on agar medium containing higher concentrations of streptomycin and the colonies developing used as two-step mutants.

Preparation of transducing phage

As reported previously (Watanabe, 1954), most of the intermediate resistant mutants are genetically unstable slow growers; fast-growing mutants arise which are less resistant to streptomycin than the original slow-growing resistant mutants. Therefore, in order to maintain the homogeneity of a population of intermediate resistant mutants the bacteria had to be subcultured on agar and fast-growing mutant colonies had to be avoided. Also when phage were to be produced on them, the slow-growing resistant cells were grown on agar plates and only the small colonies were scraped off, avoiding the areas including the large colonies. The harvested bacteria were suspended in liquid medium, infected with phage at a low multiplicity of input (10^{-3}) and aerated for 5 hr. In some of the bacterial inocula thus prepared, and also in the infected cultures thus grown, fast-growing mutants could spontaneously arise, but the presence of phage particles which carry the genome of fast-growing mutants was easily detected by infecting the original slow-growing mutants with phage stocks thus obtained and by observing if fast-growing transductants appeared. The phage stocks which contained phage particles carrying the genome of fast-growing mutants were discarded and good stocks were prepared.

It is also possible that sometimes the recipient bacteria, which were prepared by scraping off only the small colonies and suspending them in liquid medium, could contain fast-growing mutants. This possibility was checked by plating the recipient bacterial suspension on streptomycin-free agar to see if any fast-growing colonies arose. These suspensions which gave fast-growing mutant colonies on the control plates were discarded and the preparation of recipient bacteria was repeated. Even in repeated experiments some of the slow-growing resistant mutants were found to be so unstable that their transduction analysis was found to be impossible.

Transduction

In transduction of indifference or intermediate resistance into sensitive bacteria the recipient sensitive bacteria were infected with the donor phage from indifferent or intermediate resistant mutants by mixing equal volumes of bacteria and phage. As will be mentioned below, when the infected bacteria were immediately exposed to streptomycin, no transductants were recovered, showing that there is a delay for phenotypic expression in the transduction of indifference and intermediate resistance. Therefore, the following methods were used to allow the transductants to express their phenotype: the first is the delayed selection technique (Newcombe, 1952), the second being the replica plating (Lederberg & Lederberg, 1952) and the third the ordinary liquid culture method.

Delayed selection technique. 1 ml. of the infected bacteria was mixed with 9 ml. of melted agar medium in plates which were then incubated for varying periods of time after which 10 ml. of melted agar medium containing varying concentrations of streptomycin was layered on top. The plates were then kept in a refrigerator overnight to allow the streptomycin to diffuse into the lower layer. Then they were incubated at 37° for 5 days before the developed colonies were scored.

Replica plating technique. The infected bacteria were spread on the surface of agar plates, grown for varying periods of time and then replicated with velveteen pads on agar plates containing varying concentrations of streptomycin.

Liquid culture method. The infected bacteria were grown with aeration in liquid medium from which aliquots were withdrawn at various times and plated on agar containing varying concentrations of streptomycin. In contrast to the above methods, it is naturally difficult with this method to determine accurately the numbers of transductant clones, because the transductants grow mixed together with the non-transductants. In some experiments the culture of infected bacteria was allowed to undergo more divisions by diluting the culture with fresh liquid medium after it had reached saturation.

The transduction of first-step resistance into sensitive bacteria was found to be difficult, and the reason for this difficulty was assumed to be a selective disadvantage of the resistant transductants compared to wild-type recipient bacteria because of differences in their growth rates. Therefore, a slow-growing mutant, which was obtained by growing wild-type bacteria in liquid medium containing more than M/1000 $BaCl_2$ and which was sensitive to streptomycin, was also used as a recipient.

RESULTS

Characteristics of indifferent mutants from LT-2 and LT-7

As previously reported (Watanabe, 1954), the indifferent mutants of *Salmonella enteritidis*, strain no. 11, and *Escherichia coli*, strains K-12 and B-19, show generation times approximately equal to their wild-types and complete resistance to streptomycin. In LT-2 and LT-7 colonies of indifferent mutants, developed on the selective agar media containing more than 500 μg. of streptomycin per ml., were mostly composed of cells that grew rapidly when they were re-isolated on agar without streptomycin, but some were composed of fast-growing and slow-growing cells. It is not easy, however, to decide which of these two types is the primary mutant. It was found that when fast-growing mutants were grown on agar with high concentrations of streptomycin, papillae developed from which slow-growing indifferent mutants were isolated. When the slow-growing indifferent mutants were grown in the absence of streptomycin, fast-growing sectors developed from which fast-growing indifferent mutants were isolated. It seems that in the presence of high concentrations of streptomycin the slow-growing type has a selective advantage, while the situation is reversed in the absence of the antibiotic.

When phage was to be grown on a slow-growing type, the bacteria were grown on agar plates, scraped off with a loop (avoiding the areas which contained large colonies), suspended in liquid medium and then infected with phage.

Transduction of fast-growing indifference

(1) *Detection of transductants with the delayed selection technique.* As shown in Table 1, there were no indifferent transductants recovered when the infected bacteria were not allowed to divide before they were exposed to streptomycin. After 3 hr. of growth the number of visible transductant colonies decreased considerably and finally none could be detected, because the background growth became too heavy to allow the transductant colonies to be detected. Therefore, this method does not seem to be a good one for studying the kinetics of the phenotypic expression of transductants.

Table 1. *Detection of streptomycin indifferent transductants of* Salmonella typhimurium, *strains LT-2 and LT-7, with the delayed selection technique*

		Count of transductants after pre-incubation for*				
Recipient	Donor	0 hr.	1 hr.	2 hr.	3 hr.	4 hr.
Experiment 1						
LT-2 wild-type bacteria	Phage from LT-2 wild-type bacteria	0	0	0	0	0
LT-2 wild-type bacteria	Phage from LT-2 indifferent mutant	0	746	†	†	†
LT-7 wild-type bacteria	Phage from LT-7 wild-type bacteria	0	0	0	0	0
LT-7 wild-type bacteria	Phage from LT-7 indifferent mutant	0	239	†	†	†
Experiment 2						
LT-2 wild-type bacteria	Phage from LT-2 wild-type bacteria	0	0	0	0	0
LT-2 wild-type bacteria	Phage from LT-2 indifferent mutant	0	53	85	82	†
LT-7 wild-type bacteria	Phage from LT-7 wild-type bacteria	0	0	0	0	0
LT-7 wild-type bacteria	Phage from LT-7 indifferent mutant	0	19	28	17	†

* Pre-incubation means the incubation of the phage-infected bacteria in the streptomycin-free agar layer before streptomycin agar (1000μg./ml.) is added to the top.

† After 4 hr. pre-incubation, the background growth became too dense to allow visible indifferent colonies to form.

(2) *Detection of transductants with the replica-plating technique.* With this technique, it was shown again that there exists a delay in the phenotypic expression of indifferent transductants. It is probable, however, that all of the

transductant clones were not successfully replicated by this technique, because, unless the master plates were spread with so many bacteria that all of the colonies could not be successfully replicated, transductant clones were too few to be detected. Accordingly, this method is also not ideal for studying the kinetics of transduction of indifference.

(3) *Detection of transductants with the liquid culture method.* As pointed out in Methods, it is difficult to observe the individual clones of transductants with this method, but because the generation time of the indifferent transductants is fortunately about equal to that of the wild-type, the analysis of the delay of phenotypic expression of indifference seems rather easy with this method. As shown in Table 2, the frequency of indifferent transductants reaches its maximum after several generations, the minimum requirement for phenotypic expression being about one division. With this method, it is not possible to determine the numbers of divisions accurately and also there is a possibility that phage infection may take place at different times among the many recipient bacteria.

Table 2. *Detection of streptomycin indifferent transductants of* Salmonella typhimurium, *strain LT-2, with the liquid-culture method*

Duration of incubation (hr.)	Donor phage from	Viable bacteria per ml.	Indifferent bacteria per ml.*	Frequency of transductants	Factor of increase of viable bacteria
0	No phage	$3\cdot4 \times 10^8$	0	$0/3\cdot4 \times 10^8$	1·0
	Wild-type bacteria	$2\cdot4 \times 10^8$	0	$0/2\cdot4 \times 10^8$	1·0
	Indifferent mutant	$2\cdot5 \times 10^8$	0	$0/2\cdot5 \times 10^8$	1·0
1	No phage	$6\cdot0 \times 10^8$	0	$0/6\cdot0 \times 10^8$	1·8
	Wild-type bacteria	$3\cdot9 \times 10^8$	0	$0/3\cdot9 \times 10^8$	1·6
	Indifferent mutant	$3\cdot5 \times 10^8$	1	$1/3\cdot5 \times 10^8$	1·4
2	No phage	$1\cdot3 \times 10^9$	0	$0/1\cdot3 \times 10^9$	3·8
	Wild-type bacteria	$7\cdot6 \times 10^8$	0	$0/7\cdot6 \times 10^8$	3·2
	Indifferent mutant	$7\cdot7 \times 10^8$	129	$1/5\cdot9 \times 10^6$	3·1
3	No phage	$1\cdot6 \times 10^9$	0	$0/1\cdot6 \times 10^9$	4·7
	Wild-type bacteria	$8\cdot0 \times 10^8$	0	$0/8\cdot0 \times 10^8$	3·3
	Indifferent mutant	$1\cdot0 \times 10^9$	275	$1/3\cdot5 \times 10^6$	4·0
4	No phage	$2\cdot1 \times 10^9$	0	$0/2\cdot1 \times 10^9$	6·2
	Wild-type bacteria	$1\cdot0 \times 10^9$	0	$0/1\cdot0 \times 10^9$	4·1
	Indifferent mutant	$1\cdot1 \times 10^9$	663	$1/1\cdot6 \times 10^6$	4·0
5	No phage	$2\cdot4 \times 10^9$	0	$0/2\cdot4 \times 10^9$	7·1
	Wild-type bacteria	$1\cdot4 \times 10^9$	0	$0/1\cdot4 \times 10^9$	5·8
	Indifferent mutant	$1\cdot4 \times 10^9$	1280	$1/1\cdot1 \times 10^6$	5·6
7	No phage	$2\cdot8 \times 10^9$	0	$0/2\cdot8 \times 10^9$	8·2
	Wild-type bacteria	$1\cdot8 \times 10^9$	0	$0/1\cdot8 \times 10^9$	7·5
	Indifferent mutant	$1\cdot8 \times 10^9$	3990	$1/4\cdot5 \times 10^5$	7·2
16	No phage	†	—	—	—
	Wild-type bacteria	—	—	—	—
	Indifferent mutant	$7\cdot6 \times 10^8$	3220	$1/2\cdot4 \times 10^5$	3·0

* Streptomycin indifferent cells were scored on agar plates containing 500 μg. of streptomycin per ml.

† Not tested.

Lederberg (1951) reported that streptomycin resistance in *Escherichia coli*, strain K-12, is recessive to sensitivity and it is assumed to be true also in the case of *Salmonella typhimurium*, despite the contrary finding of Hotchkiss in *Diplococcus pneumoniae* (1957). However, when indifferent transductants were detected on varying concentrations of streptomycin, more transductants were recovered earlier on lower drug concentrations (Table 3), suggesting that the phenotypic expression of transduction of indifference takes place gradually from partial to complete resistance. This finding is comparable to the observation of a similar phenomenon in the transformation of streptomycin resistance in *Haemophilus influenzae* (Alexander & Leidy, 1953), and seems to suggest the participation of phenomic lag in the delay of phenotypic expression.

Table 3. *Detection of streptomycin indifferent transductants of* Salmonella typhimurium, *strain LT-2, by plating from liquid cultures on to varying concentrations of streptomycin*

Multiplicity of input	Duration of incubation (hr.)	Indifferent transductant colonies developed on agar plates containing the following concentrations of streptomycin (μg./ml.)					
		100	250	500	1000	5000	10,000
30	0	0	0	0	0	0	0
	1	0	0	0	0	0	0
	2	8	3	1	0	0	0
	3	63	26	5	4	0	0
	4	346	259	128	48	0	0
	5	69	61	62	51	39	49
	7	336	335	301	351	71	78
0·5	0	0	0	0	0	0	0
	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	10	9	3	0	0	0
	4	19	16	11	7	0	0
	6	6	7	11	10	3	3

$3\cdot4\times10^{8}$/ml. of wild-type bacteria were infected with phage from an indifferent mutant with the above multiplicities of input and aerated for varying durations after which 0·1 ml. of the undiluted culture was plated on each streptomycin agar plate.

After 4 hr. aeration of the culture, they were diluted 10 times with fresh liquid medium to allow the bacteria to continue dividing.

Essentially similar results were obtained also when donor phage was grown on slow-growing indifferent mutants, and the indifferent transductants obtained in this case were found to be all fast growing. These results seem to suggest that the slow growth of the slow-growing indifferent mutants is brought about by a secondary mutation at a locus other than the indifference locus. The experiments described below were carried out exclusively with fast-growing indifferent mutants as donors.

Other conditions of transduction of indifference

As described above, the delay in phenotypic expression is important in the transduction of indifference: besides this there are several conditions which may alter the efficiency of transduction.

(1) *Transduction with varying multiplicities of input.* When the number of recipient bacteria was kept constant and the titres of the donor phage were varied so as to give varying multiplicities of input, higher frequencies of transduction were obtained in higher multiplicities.

(2) *Transduction with varying numbers of recipient bacteria and a constant titre of donor phage.* When the titre of the donor phage was kept constant and the number of recipient bacteria was varied, it was found that the titres of the donor phage rather than the numbers of recipient bacteria determine the frequencies of transduction.

These results are comparable to those obtained by Zinder & Lederberg (1952) in the transduction of prototrophy and confirm the reasonableness of the notion of transduction per phage (Zinder, 1955).

(3) *Transduction of recipient bacteria lysogenized with homologous phage.* Wild-type bacteria were exposed to a high titre of PLT-22 phage and the survivors were repeatedly isolated on agar medium to get rid of carried-over phage. The lysogeny of the strain was proved by its immunity to a virulent mutant H-5 (obtained from Dr M. Demerec) of PLT-22 phage, the spontaneous release of the phage and ultraviolet induction of the phage. When the lysogenized bacteria were used as recipients, transduction efficiency was much lower than with the non-lysogenic recipients. Also with the virulent mutant H-5 as a donor phage, transduction with low frequency was observed in lysogenic recipient bacteria. These findings are identical to those reported by Zinder (1955) on other markers.

(4) *Cross-transduction between LT-2 and LT-7.* When the phages grown on the streptomycin indifferent mutants of LT-2 and LT-7 were used as donors and the wild-type bacteria of LT-2 and LT-7 were used as recipients, the efficiencies of transduction were about equal in the cross-transductions.

(5) *Abortive transduction.* On streptomycin agar, small colonies such as were found by Ozeki in the abortive transduction of prototrophy were looked for but none was found. This failure is not surprising, because, as mentioned above, streptomycin indifference is possibly recessive to sensitivity, and furthermore the transduction of streptomycin indifference requires a delay for its phenotypic expression. The colonies developed on streptomycin agar were found to be composed of exclusively indifferent cells.

Transduction of one-step intermediate resistance into sensitive bacteria

Phage was grown on one-step intermediate resistant mutants and sensitive recipient bacteria were infected with this phage. The above-mentioned three methods were employed to allow the possible transductants to express their phenotype but none of these methods gave significant results, because the numbers of the resistant colonies developed did not differ significantly whether phage grown on wild-type or resistant bacteria was used to infect the recipient strain.

Under this circumstance two possibilities which would interfere with the detection of transductants were investigated. One of them was the phenomenon of 'co-killing' discovered by Saz & Eagle (1953) for penicillin. Here penicillin-resistant cells are killed by penicillin when they are kept together with penicillin-sensitive cells. It was thought possible that the resistant transductants in streptomycin may be killed by subinhibitory concentrations of the antibiotic and escape detection, because they are plated with a large number of sensitive non-transductants. This possibility was examined by a reconstruction experiment, but detectable co-killing by streptomycin was not found.

The second possibility which could interfere with the detection of resistant transductants was that the resistant transductants may be selected against because of their slow growth compared to the fast growth of the sensitive non-transductants. For studying this possibility, a slow-growing mutant was obtained by growing wild-type bacteria in liquid medium containing more than M/1000 $BaCl_2$ (Watanabe, 1954). The mutant was found to be as sensitive as the wild-type to streptomycin by determining their survival curves against streptomycin. The transduction experiments in which this slow-growing mutant was used as a recipient again failed to show significant transduction. By comparing their colony sizes on agar medium this slow-growing mutant was found to become even slower growing when it became streptomycin resistant.

Transduction of second-step resistance into one-step resistant bacteria

When about 10^8 cells of a one-step resistant mutant which has the average resistance of 100 μg./ml. were plated on agar medium containing varying concentrations of streptomycin, 5 colonies developed on agar containing 1000 μg. of streptomycin per ml. One of them was used as a two-step resistant mutant. A transduction experiment was performed by using this one-step resistant mutant as a recipient and the two-step resistant mutant as a donor. The detection of two-step transductants was done with the liquid culture method; the phage-infected bacteria were plated on agar containing varying concentrations of streptomycin and their survival curves were compared with those of the control. As shown in Table 4, transduction of two-step resistance into one-step resistant cells did occur. This second-step resistance, however, might have been brought about by the mutation at the indifference locus, because negative results were obtained with several other two-step resistant strains as donors.

Transduction of first-step resistance into another one-step resistant strain

Transduction of first step into another type of one-step resistant mutant was undertaken with the liquid culture method. The results again failed to show significant differences from the control experiments. As will be reported in a following paper, genetically multiple types of one-step intermediate resistance were found by looking for wild-type, sensitive and fast-growing transductants instead of looking for resistant transductants.

Transduction of indifference into one-step intermediate resistant bacteria

A one-step mutant which is resistant to 50 μg. streptomycin/ml. was infected with phage from an indifferent mutant and the infected bacteria were aerated at 37° for 10 hr. The transductants were then detected by plating aliquots on agar medium containing 1000 μg. streptomycin/ml. The frequency of transductants per total viable bacteria was $1/4{\cdot}5 \times 10^5$ which is similar to those obtained with wild-type recipient. The indifferent transductants obtained with the one-step resistant mutant as a recipient were found to be all slow growers, even when replicated on to streptomycin-free agar medium.

Table 4. *Transduction of second step resistance into one-step intermediate resistant bacteria in* Salmonella typhimurium *strain LT-2 using the one-step mutant (resistant to 100 μg./ml.) as a recipient and the two-step intermediate-resistant mutant (resistant to 1000 μg./ml.) as a donor*

Experiment 1

Donor	Concentration of streptomycin in agar plate (μg./ml.)	No. of colonies formed on streptomycin agar plates when phage-infected cultures were aerated for the following number of hr. before plating			
		1	3	5	23
No phage	500	19	20	45	52
	1000	9	7	14	22
Phage from one-step mutant resistant to 100 μg./ml.	500	6	7	10	14
	1000	2	1	5	11
Phage from two-step mutant resistant to 1000 μg./ml.	500	3	4	10	1085
	1000	1	2	4	429

Experiment 2

Donor	Concentration of streptomycin in agar plate (μg./ml.)	No. of colonies formed on streptomycin agar plates when phage-infected cultures were aerated for the following number of hr. before plating			
		3	6	10·5	23
No phage	500	13	30	17	16
	1000	4	11	12	5
	2500	—	—	—	0
Phage from one-step mutant resistant to 100 μg./ml.	500	2	4	4	21
	1000	0	1	1	12
	2500	—	—	—	1
Phage from two-step mutant resistant to 1000 μg./ml.	500	2	5	156	452
	1000	1	1	73	265
	2500	—	—	—	8

The mixture of the equal volumes of recipient bacteria ($1{\cdot}2 \times 10^9$/ml., grown on agar plates and suspended in liquid medium to avoid large colonies) and donor phage (10^{10}/ml.) was aerated at 37° and, at various times, 0·1 ml. of the undiluted culture was plated on streptomycin agar. To the control culture equal volume of liquid medium was added instead of phage. All the plates were incubated for 5 days before the colonies were scored.

DISCUSSION

From what has been described above, it is obvious that the first report on transduction of streptomycin resistance by Zinder & Lederberg (1952) is quite reproducible when the streptomycin indifferent mutant is used as a donor and sufficient divisions are allowed for the infected bacteria to express their phenotype. The failure of some previous workers to transduce streptomycin resistance is assumed to be due to one of two reasons. Either they may have used intermediate resistant mutants as donors in which transduction is very difficult to achieve or they may not have allowed enough divisions of the infected bacteria before they were exposed to streptomycin.

The nature of the delayed expression of indifferent phenotype in its transduction has not yet been fully analysed but, from the work of Lederberg (1951), it is very likely that this delay involves a segregation lag because of the possible recessiveness of the indifference locus to its wild-type allele. If this is the case, the absence of abortive transduction is also understandable, because the cells which contain both indifferent and sensitive allelic genes should be phenotypically sensitive to streptomycin and, if they are exposed to streptomycin before the sensitive allele is segregated out by bacterial division, they should be killed by the antibiotic. Therefore, such abortive transductions as reported by Lederberg (1956), Stocker (1956) and Ozeki (1956) should not be expected to happen in this case.

Segregation lag may be a major factor of the delay, but the fact that streptomycin resistance is gradually expressed from partial to complete resistance seems to suggest the participation of phenomic lag in this delay. Unfortunately the biochemical mechanism of streptomycin action and resistance is almost unknown, although it has been investigated by Umbreit, Smith & Oginsky (1951).

One-step resistance, which is probably indifference in most cases, is the most extensively used marker in transformation experiments with *Diplococcus pneumoniae* and *Haemophilus influenzae*. Recently Corey & Starr (1957) have reported on the transformation of streptomycin resistance in *Xanthomonas phaseoli*. When we investigate the behaviour of the streptomycin resistance locus reported in these papers, we find a contradiction, i.e. in *D. pneumoniae* (Hotchkiss, 1957) this locus seems to behave as a dominant gene, whereas in *H. influenzae* (Alexander & Leidy, 1953) and *X. phaseoli* (Corey & Starr, 1957) it seems to be a recessive gene. In *Escherichia coli* it is definitely a recessive gene from the work of Lederberg (1951) and in *Salmonella typhimurium* it is very likely to be recessive from what has been reported in the present paper. It is interesting to find that a supposedly equivalent factor behaves as dominant in some species, while it acts as recessive in others; but this would be expected if one were a case of a suppressor gene.

Streptomycin indifference can be transduced not only into wild-type but also into one-step intermediate resistant bacteria. The fact that the indifferent transductants, developed from one-step intermediate resistant bacteria, are all slow growers seems to suggest that the transductants have both the one-step resistance locus and the indifference locus, and that the one-step intermediate resistance locus is epistatic to the indifference locus in controlling the slow growth, while in the control of complete streptomycin resistance the indifference locus is epistatic to the intermediate resistance locus.

In the various aspects of transduction of indifference that were also studied, this marker was found to behave in quite similar ways to other chromosomal markers.

In contrast to transduction of indifference, intermediate resistance seems to be difficult to transduce into sensitive bacteria. In many experiments there seemed to be slight increases in the numbers of the colonies developed on streptomycin plates seeded with sensitive bacteria infected with phage from a resistant mutant over those of the control experiments in which either the phage from wild-type bacteria or no phage was added to the recipient bacteria. Sometimes, on the other hand, there were no differences found.

However, the transduction of a second step into one-step resistant cells did occur successfully. The long delay before the resistant transductants were detected possibly indicates that multiple-step streptomycin resistance is recessive as is streptomycin indifference. In this transduction experiment, however, the second step mutation might have been brought about by the mutation at the indifference locus. Furthermore, in none of the tested combinations of one-step resistant mutants as recipients and donors, did a significant transduction to higher resistance occur.

One may argue that intermediate resistance might be inherited by cytoplasmic factors, but the spontaneous development of fast-growing mutants with lower levels of resistance and the consistent and reproducible transduction of wild-type alleles into the resistance loci which will be reported in a following paper seem to make this assumption unlikely. For a conclusive proof of the chromosomal nature of the control of resistance, the discovery of linkage of resistance loci to other chromosomal markers is felt necessary. Studies along this line are now in progress, but so far no linkage relationship has been found. The failure to obtain two-step resistant transductants by crossing one-step resistant mutants with other types of one-step resistant mutants may indicate that the loci which control first-step resistance are different in action from those which control second steps.

The reason why one-step resistance is not easily transduced into sensitive bacteria is not clear as yet; it is assumed that transduction of resistance does take place, but that the resistant transductants are possibly selected against by the sensitive-non-transductants due to differences in their growth rates. It was shown that the difficulty of detecting resistant transductants is not due to 'co-killing' and the failure even with a streptomycin-sensitive slow-growing recipient suggests a need for improving the technique.

Before the transduction of resistance becomes reproducible with some technical improvement, it may be premature to discuss the failure to obtain two-step resistant transductants by crossing two one-step resistant mutants by transduction. At least at the present time it is not possible to analyse the genetics of intermediate resistance by crossing one-step resistants and looking for higher resistance.

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Transduction of Streptomycin Sensitivity into Resistant Mutants of *Salmonella typhimurium*

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SUMMARY: The infection of slow-growing one-step intermediate streptomycin-resistant mutants with phage from wild-type bacteria, indifferent (one-step completely resistant) or other one-step intermediate resistant mutants gives rise to fast-growing streptomycin-sensitive transductants. From this fact it is clear that both slow growth and streptomycin resistance are controlled by a single locus. The transductants are very easily detected on the poor background growth of the intermediate resistant recipient. With this technique it has become possible to cross one-step resistance with resistance, indifference and sensitivity. The results so far obtained indicate that one-step intermediate resistance is controlled by multiple loci. None of these loci is linked to the indifference locus. The fast-growing mutants which develop from the two-step resistant mutants are assumed from the transduction analysis to be due to reverse mutations at the first step loci.

In a preceding paper Watanabe & Watanabe (1959) reported that streptomycin indifference (one-step complete resistance) is easily transduced into sensitive or intermediate resistant bacteria. On the other hand, the transduction into sensitive recipients of intermediate resistance obtained by one-step and two-step selection is not easily detected, even when the delay in phenotypic expression is taken fully into consideration. None the less, sensitive alleles can be transduced into intermediate resistant mutants as will be reported in the present paper.

Since it was established that streptomycin indifference is controlled by a single locus (Watanabe, 1954), it is easily assumed that the streptomycin sensitive allele will also be transduced into indifferent mutants, although on account of the lack of proper selective methods it may not be easy to detect the sensitive transductants among the indifferent cells. On the other hand, as described before (Watanabe, 1954; Watanabe & Watanabe, 1959), all of the intermediate streptomycin-resistant mutants are slow growers, and from them fast-growing sensitive or less resistant mutants come out spontaneously. Indeed, in the case of one-step intermediate resistant cells infection with phage from wild-type bacteria was found to give rise to fast-growing streptomycin sensitive transductants. This finding has led us to the transductional analysis of various types of streptomycin resistant mutants by looking for wild-type transductants rather than by looking for resistant transductants.

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METHODS

The system of *Salmonella typhimurium*, strain LT-2, and temperate phage PLT-22 was used exclusively. The indifferent mutants were those which were described in a preceding paper (Watanabe & Watanabe, 1959). For detecting the sensitive transductants among the indifferent mutants, streptomycin-free agar plates seeded with about 500 infected bacteria on their surface were replicated on agar plates containing 500μg./ml. of streptomycin and colonies on the master plates which did not give resistant colonies on replica plates were looked for.

In order to isolate independent intermediate resistant mutants, liquid medium containing about 100 cells of wild-type bacteria per ml. was distributed into many test tubes in 1 ml. amounts. After incubation for 18 hr. at 37°, 0·1 ml. of each culture was plated on agar medium containing 25, 50, 100 and 250μg. streptomycin/ml. The inoculated plates were incubated at 37° for 5 days and three colonies were picked at random from each plate and purified by repeating single colony isolations on streptomycin-free agar. Plates which contained 250μg. streptomycin/ml. developed few or no colonies. The mutants thus obtained were used as one-step resistant mutants; the method of isolating two-step resistant mutants has been described elsewhere (Watanabe & Watanabe, 1959). In transduction experiments with intermediate resistant bacteria, previously mentioned precautions were taken to avoid spontaneous fast-growing mutants (Watanabe & Watanabe, 1959).

The media used have also been described in the same publication.

RESULTS

Transduction of sensitivity into indifferent bacteria

As reported in a preceding paper (Watanabe & Watanabe, 1959), indifference from both fast- and slow-growing strains can be transduced into sensitive bacteria with about equal frequencies and the obtained indifferent transductants are all fast growers in both cases. In an attempt to transduce sensitivity into indifferent mutants, fast-growing indifferent mutants were first used and possible sensitive transductants were looked for with the replica plating technique. More than 10,000 colonies, developing on master plates from indifferent bacteria infected with phage from wild-type bacteria at multiplicities of input 5 and 100 were replicated on streptomycin agar plates. None of them was found to be sensitive to streptomycin. The frequency of transduction of sensitivity into indifferent bacteria seems, therefore, to be lower than 10^{-4}, if there is any such transduction. This fact is not surprising from experience with the transduction of other markers. It may be possible to discover sensitive transductants, if very many more plates are replicated in the above way.

Next, slow-growing indifferent mutants were used as recipients. When large numbers of recipient bacteria infected with phage from wild-type bacteria were plated on agar plates without streptomycin, it was found that fast-growing colonies developed on the poor background growth. They were replicated on agar plates containing 1000 μg. streptomycin/ml. and more than 500 colonies replicated were all found to be resistant to streptomycin at this concentration. From this result it will be understood that the slow growth and the indifference of the slow-growing indifferent mutants are possibly controlled by two different loci. Thus the detection of sensitive transductants cannot be facilitated by using slow-growing indifferent mutants as recipients and looking for large colonies, because in these large colonies only the locus which controls slow growth seems to have been replaced by transduction.

Transduction of sensitivity into one-step intermediate resistant bacteria with phage from wild-type bacteria

The infection of one-step resistant mutants with phage from wild-type bacteria gave large colonies on a background of poor growth; whereas control plates spread with bacteria infected with homologous phage and uninfected by phage gave no large colonies. In some experiments the control plates gave some large colonies of spontaneous fast-growing mutants; these experiments were repeated.

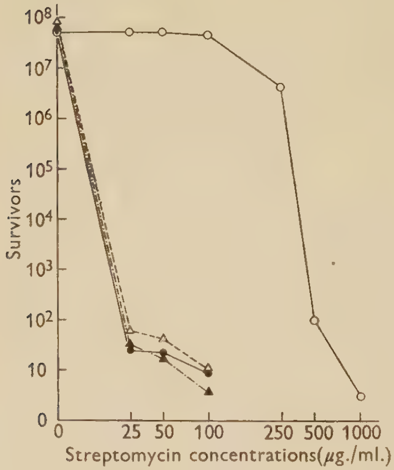

Fig. 1

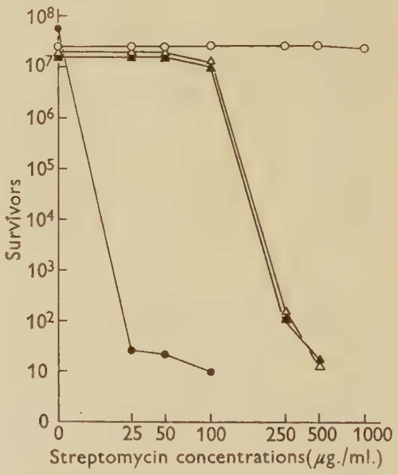

Fig. 2

Fig. 1. Survival curves, in streptomycin, of *Salmonella typhimurium*, strain LT-2 wild-type, the one-step resistant mutant A, fast-growing transductants developed from mutant A with phage from wild-type bacteria. Varying dilutions of each bacterial suspension were plated on agar medium containing varying concentrations of streptomycin and the colonies developed were scored after 5 days' incubation of the inoculated plates. ○—○, Mutant A; △---△, wild-type transductant no. 1; ▲---▲, wild-type transductant no. 2; ●—●, wild-type.

Fig. 2. Survival curves, in streptomycin, of *Salmonella typhimurium*, strain LT-2 wild-type, the two-step resistant mutant B/1000, fast-growing mutant and transductant derived therefrom. Varying dilutions of each bacterial suspension were plated on agar medium containing varying concentrations of streptomycin and the colonies developed were scored after 5 days' incubation of the inoculated plates. ○—○, Mutant B/1000; △—△, fast-growing transductant; ▲—▲, fast-growing mutant; ●—●, wild-type.

In studying the transduction of sensitivity into one-step intermediate resistant bacteria, it was found that the fast-growing transductants were invariably as sensitive to streptomycin as the wild-type bacteria. This was found with replica plating as well as survival curve determinations (Fig. 1). In addition, spontaneous fast-growing mutants developed from one-step resistant mutants were also found to have a sensitivity to streptomycin equal to wild-type bacteria. These facts seem to suggest that both the slow growth and the streptomycin resistance of the one-step intermediate resistant mutant are controlled by a single locus. For this reason the fast-growing sensitive transductants are called wild-type transductants.

The frequencies of wild-type transductants varied considerably according to the recipient when phage from wild-type was used as a donor as shown in Table 1. Some of the one-step intermediate resistant mutants were found to give only small numbers of wild-type transductants.

Table 1. *Transduction of sensitivity into one-step intermediate streptomycin resistant bacteria in* Salmonella typhimurium, *strain LT-2, with phage grown on wild-type and indifferent bacteria*

	No. of wild-type transductants in 0·1 ml. of phage-infected bacteria	
Recipient	With phage from wild-type bacteria	With phage from indifferent bacteria
100M (100 μg./ml.-resistant)	3	2
250C (250 μg./ml.-resistant)	4	6
25–1A (25 μg./ml.-resistant)	25	23
50–2B (50 μg./ml.-resistant)	46	33
250–2A (250 μg./ml.-resistant)	3	5
100–3A (100 μg./ml.-resistant)	48	48
50–5A (50 μg./ml.-resistant)	34	35
25–7A (25 μg./ml.-resistant)	26	25
25–8A (25 μg./ml.-resistant)	31	26
50–9B (50 μg./ml.-resistant)	26	58
25–10A (25 μg./ml.-resistant)	26	40
50–12A (50 μg./ml.-resistant)	360	320
25–13A (25 μg./ml.-resistant)	25	33
100–14A (100 μg./ml.-resistant)	200	178

Equal volumes of a recipient bacterial suspension (about 10^9/ ml.) and phage (10^{10}/ml.) from either wild-type or indifferent bacteria were mixed and aerated at 37° for 10 min., when 0·1 ml. of undiluted infected culture was plated on agar without streptomycin. Large colonies were counted after incubating the plates for 2 days.

Transduction of sensitivity into one-step resistant bacteria with phage from indifferent mutants

All strains of intermediate resistant bacteria infected with phage grown on fast-growing as well as slow-growing indifferent mutants gave numbers of wild type transductants approximately equal to the numbers found when phage from wild-type bacteria was used as shown in Table 1. None of the fast-growing transductants was found to be resistant to streptomycin. Therefore, the loci which control one-step intermediate resistance are not closely linked to that of streptomycin indifference.

Table 2. *Transduction of sensitivity into one-step intermediate streptomycin resistant mutants of* Salmonella typhimurium, *strain LT-2, with phage grown on the one-step intermediate resistant mutant*

	No. of wild-type transductants in 0·1 ml. of phage-infected bacteria	
Recipient	When infected with phage from mutant B	Control with no phage
A	18	0
100–1 B	281	0
25–1 C	116	0
100–1 C	45	0
50–1 A	0	0
50–1 B	98	85
50–1 C	0	0
25–2 A	*c.* 1000	0
50–2 A	44	10
50–2 C	18	0
100–2 C	8	0
25–3 A	0	0
50–3 A	242	0
25–3 C	0	0
50–3 C	40	0
250–3 A	457	2
100–3 B	55	0
25–4 A	0	0
100–4 A	17	11
25–4 B	279	0
100–4 B	12	1
25–4 C	63	0
50–4 A	*c.* 1000	0
50–4 B	210	0
50–4 C	146	0
25–5 B	3	0
25–5 C	0	0
25–6 A	12	0
50–6 C	11	0
100–6 A	157	18
100–6 C	78	22
*25–7 A	88 (26)	1
25–7 B	93	0
25–7 C	86	0
50–7 A	80	0
50–7 B	81	0
*25–8 A	75 (31)	0
100–8 C	0	0

* These are the only strains also in Table 1 (wild-type transductants in parentheses).

Equal volumes of phage grown on one-step mutant B (100 μg./ml.-resistant) (10^{10}/ml.) and bacterial suspension (about 10^9 cells/ml.) were mixed and aerated at 37° for 10 min., when 0·1 ml. of the phage-infected bacterial suspension was spread on agar without streptomycin. For the control experiment, an equal amount of liquid medium was added instead of phage. Large colonies were counted after incubating the plates for 2 days.

Transduction of sensitivity into one-step intermediate resistant bacteria with phage grown on other intermediate resistant mutants

When many independently isolated mutant strains of one-step intermediate resistance were infected with a phage stock grown on a certain strain of one-step intermediate resistance, it was found that some of the strains gave various numbers of wild-type transductants, while some gave none (Table 2). The mutant strains which gave no wild-type transductants may be grouped together and the mutant strains which gave wild-type transductants may be put into different groups. With this grouping several classes can be found among the relatively stable one-step intermediate resistant mutants. A part of reciprocal transduction results among one-step intermediate resistant mutants of different groups is shown in Table 3.

Transduction of sensitivity into one-step intermediate resistant bacteria with phage from two-step intermediate resistant mutants

When two-step intermediate resistant mutants were derived from one-step resistant mutants, they were invariably slow growers like the one-step mutants. The infection of one-step intermediate resistant mutants with phage from the two-step intermediate resistant mutants which are supposed to have the same first step was found to give no fast-growing transductants as shown in Table 4. However, when phage from a two-step resistant mutant which was derived from one-step resistant bacteria of a different group was used as a donor, many fast-growing transductants were obtained and they were all found to be sensitive to streptomycin as seen in this table.

Table 3. *Reciprocal transduction of sensitivity into one-step streptomycin resistant mutants among independently isolated mutant strains of* Salmonella typhimurium, *strain LT*-2

	No. of wild type transductants developed with donor phage grown on		
Recipient	50–4 B	50–8 A	25–12 A
50–4 B (50 μg./ml.-resistant)	0	68	105
50–8 A (50 μg./ml.-resistant)	8	0	158
25–12 A (25 μg./ml.-resistant)	87	329	0

Equal volumes of donor phage (10^{10}/ml.) and recipient bacteria (about 5×10^9/ml.) were mixed and aerated at 37° for 10 min., when 0·1 ml. of the infected bacterial suspension was plated on agar without streptomycin. Large colonies were counted after incubating the plates for 2 days.

Table 4. *Transduction of sensitivity into one-step intermediate streptomycin resistant mutant of* Salmonella typhimurium, *strain LT*-2, *with phage grown on two-step resistant mutants*

Donor	No. of wild-type transductants in 0·1 ml. of phage-infected bacteria
None	0
Phage from wild-type bacteria	782
Phage from one-step mutant B (100 μg./ml.-resistant)	0
Phage from two-step mutant B/1000 (1000 μg./ml.-resistant)	0
Phage from one-step mutant C (250 μg./ml.-resistant)	810
Phage from two-step mutant C/1000 (1000 μg./ml.-resistant)	980

Equal volumes of phage (10^{10}/ml.) and suspension (about 10^9 cells/ml.) of one-step mutant B (100 μg./ml.-resistant) were mixed and aerated at 37° for 10 min., when 0·1 ml. of the phage-infected bacterial suspension was spread on agar without streptomycin. Large colonies were counted after incubating the plates for 2 days.

Mutants B and C were found by transduction to belong to different groups.

Transduction of fast growth into two-step resistant bacteria with the phage either from wild-type bacteria, one-step resistant, indifferent or spontaneous fast-growing mutants

As shown in Table 5, all of the phage from wild-type, one-step resistant, indifferent and spontaneous fast-growing mutants were found to give fast-growing transductants with about equal frequencies. The fast-growing transductants developed from two-step resistant bacteria, however, were found not to be sensitive to streptomycin but still of intermediate resistance (Table 5 and Fig. 2). We have previously reported (Watanabe, 1954) that the fast-growing mutants which developed spontaneously from multiple-step resistant mutants are less resistant to streptomycin than their parents but not as sensitive as the wild-type. The transduction experiment described here seems to indicate that the fast-growing intermediate resistant transductants derived from the two-step mutants have recovered fast growth by the replacement of the first-step mutant locus with its wild-type allele, although they still retain the second-step mutant allele. If this is the case, a question may come up why one does not find this second step occurring as an independent mutation, i.e. a first-step, fast-growing mutant. Actually this type of mutant is not isolated, suggesting another possibility, i.e. the participation of suppressor mutations. For testing this possibility, the spontaneous fast-growing mutants developed from two-step resistant mutants were infected with phage from wild-type bacteria to see if any transductants with higher resistance came out by replacing the suppressor locus with its wild-type allele. In repeated experiments with several strains, however, no positive results were obtained. These

Table 5. *Transduction of fast growth into two-step intermediate streptomycin resistant bacteria of* Salmonella typhimurium, *strain LT-2, with phage grown on either wild-type bacteria, one-step intermediate resistant, indifferent or spontaneous fast-growing mutants*

Donor	No. of fast-growing transductants in 0·1 ml. of phage-infected bacteria	No. of large colonies on replica plates containing streptomycin (μg./ml.) 25	100
None	0	0	0
Phage from wild-type bacteria	834	834	108
Phage from two-step mutant B/1000 (1000 μg./ml.-resistant)	0	0	0
Phage from one-step mutant C (250 μg./ml.-resistant)	920	920	135
Phage from fast-growing mutant from B/1000	940	940	189
Phage from indifferent mutant	1080	1080	156

Equal volumes of phage (10^{10}/ml.) and suspension (about 10^9 cells/ml.) of two-step mutant B/1000 (1000 μg./ml.) were mixed and aerated at 37° for 10 min., when 0·1 ml. of the phage-infected bacterial suspension was spread on agar without streptomycin. Large colonies were counted after incubating the plates for 2 days.

negative data may indicate that at least such suppressors that are not closely linked to the resistance loci are not concerned with the decrease of resistance and recovery of fast growth in multiple-step resistant mutants.

Nutritional requirements of one-step intermediate resistant mutants

Although no auxotrophs were found among the intermediate streptomycin resistant mutants of *Salmonella enteritidis*, strain no. 11, and *Escherichia coli*, strains B-19 and K-12, despite the fact that they are invariably slow growers on complete media (Watanabe, 1954), some of the one-step intermediate resistant mutants of *S. typhimurium*, strain LT-2, were found to be auxotrophs. Furthermore, all of them were found to require both thiamine and nicotinic acid for their growth. Some of them are quite stable, while the others are fairly mutable. By transduction with phage grown on wild-type bacteria, this type of mutant is changed in a single step to wild-type which is fast growing, streptomycin sensitive and prototrophic; these characters never segregate from each other. Since the mutants of this group seem to be of particular interest from both genetic and biochemical viewpoints, their transductional and biochemical studies will be reported separately in a following paper.

DISCUSSION

In a preceding paper (Watanabe & Watanabe, 1959), it was reported that streptomycin indifference (one-step complete resistance) is easily transduced, whereas intermediate resistance is not easily transduced. The apparent difficulty of transducing resistance into sensitivity even suggests the possibility that intermediate streptomycin resistance might be controlled by some cytoplasmic factors. If the loss of these factors were to cause the loss of streptomycin sensitivity, that is the development of streptomycin resistance, it is not surprising that streptomycin resistance is not transduced. In fact Sager (1954) reported that one type of streptomycin resistance in *Chlamydomonas reinhardi* is controlled by cytoplasmic factors.

The success of transduction of sensitive wild-type alleles into one-step resistant bacteria, however, seems to make the possibility of the participation of cytoplasmic inheritance of streptomycin resistance unlikely confirming the conclusion to be drawn from the fact that there are frequent fast-growing streptomycin sensitive spontaneous mutants. These spontaneous mutants of wild-type phenotype were found to have the wild-type allele at the locus of first-step resistance by transduction experiments in that the phage from these spontaneous mutants was found to be able to transduce fast growth and streptomycin sensitivity into one-step resistant bacteria in a single step. Before we succeed in showing that the loci for streptomycin resistance are linked to some other chromosomal markers, it is not possible to draw a decisive conclusion about the chromosomal nature of streptomycin resistance.

We have pointed out before (Watanabe, 1954) that streptomycin indifference is a rather exceptional type of resistance to antibiotics; the usual feature of drug resistance is quantitative variation. Demerec (1948) assumed that both streptomycin and penicillin resistance are controlled by multiple genes but in his later work (Demerec, 1950) he reported that streptomycin resistance is controlled by a single locus or by very closely linked loci.

The fact that the infection of slow-growing indifferent mutants with phage from wild-type gives fast-growing indifferent transductants seems to show that the slow growth of this type of indifferent mutant is due to a secondary mutation at a locus other than the indifference locus. Furthermore, the infection of wild-type bacteria with phage from slow-growing indifferent mutants was found to give fast-growing indifferent transductants.

The transduction of fast growth and streptomycin sensitivity into one-step intermediate resistant bacteria with phage from wild-type bacteria, indifferent mutants or other intermediate resistant mutants indicates that both slow growth and one-step intermediate streptomycin resistance are controlled by a single locus. The fact that the replacement of the first-step resistance locus of two-step resistant bacteria with its wild-type allele gives fast-growing intermediate resistant transductants shows that the second-step resistance locus may control only resistance and not slow growth. Although much attention has to be paid to the fact that fast-growing, intermediate streptomycin resistant mutants are not isolated by one-step selection with streptomycin, the failure to prove suppressor mutations for the decrease of resistance and recovery of fast growth and also the failure to obtain transductants of higher resistance by crossing one-step intermediate resistant mutants of different groups (Watanabe & Watanabe, 1959) seem to support this assumption.

The transduction studies reported in the present series clearly demonstrate that streptomycin resistance is controlled by multiple loci which are not linked to the locus of streptomycin indifference. The transduction of first step into other one-step resistant bacteria does not succeed as mentioned above and it is not known at the present time whether or not this failure is due to some technical reason. If it is not transduced even after technical improvements, it may mean that the first-step mutations are different in action from the second steps and there may be many second steps, third steps and so on. In fact, as discussed above, there is some indication that the second steps may be different from the first steps.

Streptomycin resistance has been most extensively studied among various kinds of bacterial drug resistances and streptomycin-resistant mutants have been used in various experiments. For example, streptomycin-resistant mutants were used to study the mode of action of streptomycin (Umbreit, Smith & Oginsky, 1951) and streptomycin resistance has been a favourite marker in studying the kinetics of transformation. Although most of the streptomycin resistant mutants used in these studies may be assumed to be streptomycin indifferent from the way they were described to behave, some of them may have been intermediate ones, some of which are one-step and others multiple step. It is evident from the present studies that streptomycin indifference behaves in a different way from intermediate resistance and intermediate resistance can be grouped genetically into multiple classes. It would not be surprising, therefore, if they would behave in different ways from each other and if their biochemical mechanisms were different. Accordingly, studies which employ different mutants may produce different results.

Newcombe & Nyholm (1950) and Demerec (1950) reported that streptomycin dependence is alleleic to complete resistance. In the present studies the isolation of dependent mutants was attempted in order to study their alleleic relation to indifference by transduction but the mutation rates of strains LT-2 and LT-7 to streptomycin dependence seem to be extremely low. From more than 10 cultures and from more than 200 colonies which were completely resistant to streptomycin, no dependent colonies were recovered. Hashimoto (personal communication) found that the infection of dependent mutants of *Escherichia coli* strain B with P1 phage from indifferent mutants gave non-dependent transductants on agar without streptomycin, and most of these transductants were found to be completely resistant (indifferent) to streptomycin. His results seem to confirm the previous conclusions of Newcombe & Nyholm (1950) and Demerec (1950).

We are very grateful to Dr Francis J. Ryan for generously giving us a chance of working in his laboratory, for valuable suggestions and discussions in conducting this work and also for much help in writing the manuscript. Our thanks are also due to Dr M. Demerec for kindly supplying his strains.

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Kinetics of Bacterial Growth: Relationship between Population Density and Specific Growth Rate of Continuous Cultures

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SUMMARY: Results from studies of continuous cultures of *Aerobacter aerogenes* growing in chemically defined media indicate that specific growth rate (R) is a function of population density (P) as well as the concentration of the limiting nutrient (S). From these observations, and those of others, the following model for bacterial growth is derived:

$$R = \frac{u_m S}{BP+S},$$

where u_m and B are growth parameters that are constants under defined conditions. This model is believed to have general applicability and to account for bacterial growth in both batch and continuous cultures.

Although biologists have long recognized that the specific growth rate of an organism is often a function of its population density (Pearl & Parker, 1922; Greenleaf, 1926), most bacteriologists are of the opinion that the specific growth rate of a population of bacteria is, ideally, independent of population density. This idea has come down into recent treatments of bacterial growth kinetics of both batch and continuous cultures; and most workers have regarded the specific growth rate of a population growing under conditions of nutrient limitation as a function only of the concentration of the limiting nutrient (Monod, 1942, 1950; Novick & Szilard, 1950; Spicer, 1955; Herbert, Elsworth & Telling, 1956; Moser, 1958). Nevertheless, evidence supporting the contention that specific growth rates of bacterial populations are also functions of population density may be found in the old theories and concepts of 'cell space' (Bail, 1929). Contois (1957) presented evidence to this effect from studies of batch cultures growing under conditions of nutrient limitation. This paper is a report upon further studies undertaken to ascertain the effect of population density upon specific growth rates of bacterial populations, and as a further attempt to derive a more satisfactory model for bacterial growth.

METHODS

Organism. A strain of *Aerobacter aerogenes* was used which grew well in mineral salts media containing ammonium as a nitrogen source and glucose or succinate as a carbon source. In each batch of medium, either the nitrogen source or the carbon source was limiting, all other components being present in excess.

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Culture media. These were compounded from the following stock aqueous solutions:

Solution A, glucose 200 mM.

Solution B, succinic acid 200 mM.; neutralized to pH 6·8 with KOH.

Solution C. $NH_4H_2PO_4$ 250 mM.; $(NH_4)_2HPO_4$ 250 mM.

Solution D. $MgSO_4.7H_2O$ 80 mM.; $FeSO_4.7H_2O$ 3·5 mM.; $MnCl_2.4H_2O$ 5·0 mM.; HCl 1·0 mM.

Solution E. Na_2HPO_4 250 mM.; KH_2PO_4 250 mM.

Solution F. Tetrasodium salt of ethylenediaminetetraacetic acid 25 mM.

All media contained 10 ml. solution D and 5·0 ml. solution F/1. The volumes of solutions C and A or B were changed from time to time to give desired concentrations of ammonium and the selected carbon source. Once the volume of solution C was determined for a given batch or medium, a volume of solution E was added that was sufficient to give a final total phosphate concentration of M/30.

Media were sterilized by passage through sterile molecular filters (Millipore type PH, available from Millipore Filter Corp., Watertown 72, Massachusetts, U.S.A.) and dispensed by the usual aseptic techniques. Before inoculation, the pH value of all media was 6·8.

Method of cultivation. A continuous culture device similar in theory and design to the one developed by Novick & Szilard (1950) was used. The flow rate was adjusted by means of a stopcock which controlled the rate at which fresh medium dripped into the culture vessel. Flow rates were estimated by measuring the volumes of effluent accumulating over known time intervals as well as by drop counts of the inflowing medium.

Culture volume was constant for any given culture, but was different from time to time depending upon the particular culture vessel used. All culture volumes were between 30 and 45 ml.

Mixing and aeration of cultures was accomplished by vigorous sparging with sterile air at a rate of *c.* 1·0 l. air/ml. culture volume/hr.; and the culture device was designed so that the inflowing medium was blown into the culture by the stream of sterile air. In addition, two sterile buoyant rubber disks *c.* 8 mm. in diameter were placed in each culture vessel. By their continuous rapid movement throughout the culture, they scrubbed the walls of the culture vessel and eliminated wall-growth completely. They were also self-cleansing.

Measurement of population density. Population density was measured in units of optical density by a Beckman model DU spectrophotometer (available from Beckman Instruments, Inc., Fullerton, California, U.S.A.) with 1·0 cm. Pyrex cuvettes and at 420 mμ. Comparisons of optical densities with direct counts showed that an optical density of 1·0 corresponded to a direct count of *c.* 5×10^8 organisms/ml. Experience showed that optical density was a linear function of population density within values of optical density of 0·0–0·6. In practice, a sample of a culture was removed aseptically from the culture vessel and diluted until its optical density was less than 0·4 unit. The resultant reading was multiplied by the dilution factor to give the correct population density in units of optical density.

Estimation of concentration of limiting nutrient. Previous experience (Contois, 1957) had shown that with this organism and these media the uptake of the limiting nutrient was a linear function of the increase in population density, or

$$c(S_0 - S) = (P - P_0), \tag{1}$$

where S_0 is the initial concentration of the limiting nutrient, P_0 is the initial population density, and c is a yield coefficient. Studies by Dagley & Hinshelwood (1938), Lodge & Hinshelwood (1939), Monod (1942), Herbert *et al.* (1956) and Lawrence (1957) have shown that this relationship is valid under appropriate conditions with various limiting nutrients serving as either carbon, nitrogen, phosphorus, or sulphur sources.

In continuous cultures, S_0 is the concentration of the limiting nutrient in the inflowing medium. Since P_0 is usually insignificant and is washed out of the culture in time anyway (see Spicer, 1955) equation (1) reduces to

$$cS = (cS_0 - P), \tag{2}$$

where cS_0 is equivalent to the maximum population density that an organism can attain in a particular medium.

In this study, estimates of the concentration of the limiting nutrient (S) were calculated by equation (2) from measurements of population density (P), the maximum attainable population density (cS_0), and the known values of S_0. In some of the cultures in which the nitrogen source was limiting, its concentration also was measured directly. This was done by removing a sample from the culture vessel and diluting it 1/10 with distilled water. The mixture was filtered immediately through a molecular filter (Millipore type PH) and a sample of the filtrate diluted and nesslerized. After sufficient time, colour development was measured by a Beckman DU spectrophotometer in 10 cm. Pyrex cuvettes at 476 mμ. Estimates of the concentration of ammonium were obtained by comparison with standards.

Estimation of specific growth rates. Monod (1950) and Novick & Szilard (1950) showed that in continuous cultures the change in population density in time is equal to the rate at which the population grows minus the rate at which it is washed out of the culture vessel, or

$$\frac{dP}{dt} = RP - DP, \tag{3}$$

where R is the specific growth rate and D, dilution rate, is defined as the rate of flow of medium through the culture vessel per unit volume of culture (see Herbert *et al.* 1956).

In these experiments, it was desired to obtain estimates of specific growth rate and concentration of limiting nutrient when cultures were at equilibrium. Under such conditions, dP/dt of equation (3) is zero and R is equal to D. Estimates (in units of hr.$^{-1}$) of specific growth rate, then, were made from measurements of flow rate and the known culture volumes. Replicate estimates were found to have a standard error of *c.* 2 % of the mean of the estimates.

Other observations. Periodic measurements of the pH value of cultures were also made as an aid to assuring that any drastic changes in environmental conditions would be observed. In addition, samples of cultures were examined from time to time during each experiment for the purity of the bacterial species being grown. This was done by microscopic examinations of stained preparations and by plating samples into differential media.

RESULTS

After sterilization, the apparatus was assembled, connected to a reservoir of sterile medium, and placed in an incubator at 27°. The culture vessel was filled with medium and inoculated from a stock culture of *Aerobacter aerogenes*. After growth had proceeded for several hours, fresh medium was allowed to flow through the culture vessel at a constant rate. Determinations of dilution rate (D) and population density were made periodically. After a time, the population density reached a constant value, indicating that equilibrium had been established. After several determinations of the equilibrium population density (P_e), the flow rate was adjusted to a new constant value and P_e was again determined. Two typical series of such measurements are shown in Fig. 1. By these methods, values of R and their corresponding values of P_e were obtained for each culture (cf. Herbert *et al.* 1956).

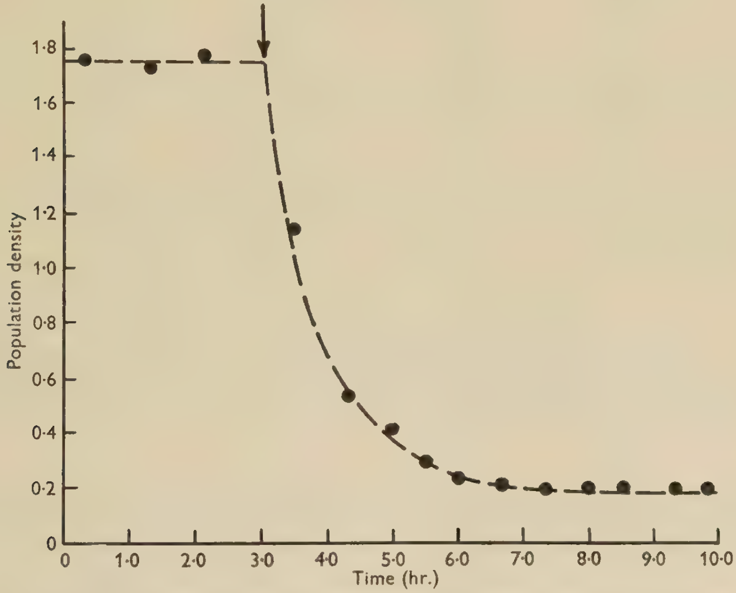

Fig. 1. Change in population density with change in dilution rate of a continuous culture of *Aerobacter aerogenes* with glucose limiting. Dilution rate changed from 0·50 to 1·05 hr.$^{-1}$ at point indicated by arrow after culture had been in equilibrium for *c.* 17 hr.

Estimates of cS_0 were obtained by stopping the flow of medium and allowing the culture to attain its maximum population density at various times during each experiment.

Table 1. *Relationship between specific growth rate* (R), *population density* (P_e), *and concentration of limiting nutrient* (S_e) *in continuous cultures of* Aerobacter aerogenes *at equilibrium**

R	P_e	(cS_0-P_e)†	S_e‡
Culture 1A. Glucose as the carbon source and ammonium as the nitrogen source. Glucose limiting at a concentration of 5·55 mM. in the inflowing medium.			
1·07	0·49	2·03	4·48
0·97	1·83	0·69	1·53
0·74	2·20	0·32	0·71
0·49	2·37	0·15	0·34
0·46	2·40	0·12	0·25
0·00	2·52	0·00	0·00
Culture 1B. Glucose as the carbon source and ammonium as the nitrogen source. Ammonium limiting at a concentration of 3·75 mM. in the inflowing medium.			
1·01	0·35	1·66	3·10
0·83	0·71	1·30	2·42
0·79	1·14	0·87	1·62
0·44	1·78	0·23	0·43
0·29	1·83	0·18	0·33
0·00	2·01	0·00	0·00

* Values of R and P_e are averages of several determinations.

† This term is an estimate of the concentration of the limiting nutrient in units of population density. The quantity cS_0 is constant for each culture and equal to the value of P_e at R equal to zero.

‡ Estimates of this term were calculated by means of equation (2) in the text and are expressed as millimoles per l.

Table 2. *Relationship between specific growth rate* (R), *population density* (P_e), *and concentration of limiting nutrient* (S_e) *in continuous cultures of* Aerobacter aerogenes *at equilibrium**

R	P_e	(cS_0-P_e)†	S_e‡
Culture 2A. Succinic acid as the carbon source and ammonium as the nitrogen source. Succinic acid limiting at a concentration of 8·47 mM. in the inflowing medium.			
0·45	0·78	1·17	5·07
0·22	1·71	0·25	1·07
0·21	1·65	0·30	1·31
0·17	1·74	0·21	0·93
0·00	1·95	0·00	0·00
Culture 2B. Succinic acid as the carbon source and ammonium as the nitrogen source. Succinic acid limiting at a concentration of 16·95 mM. in the inflowing medium.			
0·52	0·15	3·86	16·31
0·45	1·18	2·83	11·96
0·42	2·17	1·84	7·78
0·19	3·63	0·38	1·61
0·00	4·01	0·00	0·00

* Values of R and P_e are averages of several determinations.

† This term is an estimate of the concentration of the limiting nutrient in units of population density. The quantity cS_0 is constant for each culture and equal to the value of P_e at R equal to zero.

‡ Estimates of this term were calculated by means of equation (2) in the text and are expressed as millimoles per l.

Data from several experiments are given in Tables 1–3 and the relationships between R and P_e for each experiment are plotted in Fig. 2.

In cultures 3A and 3B (data of Table 3), determinations of the concentration of the limiting nutrient (ammonium) were made by two independent methods: (*a*) by means of equation (2) from estimates of P_e and cS_0; and (*b*) direct measurement of the concentration of ammonium. Figure 3 shows plots of estimates made by these two methods. The results confirm the relationship defined by equation (2) and show that the yield coefficient (the slope of the line in Fig. 3) is independent of specific growth rate.

Table 3. *Relationship between specific growth rate* (R), *population density* (P_e), *and concentration of limiting nutrient* (S_e) *in continuous cultures of* Aerobacter aerogenes *at equilibrium**

R	P_e	(cS_0-P_e)†	S_e‡
Culture 3A. Succinate as the carbon source and ammonium as the nitrogen source. Ammonium limiting at a concentration of 3·75 mM. in the inflowing medium.			
0·54	0·37	1·77	—
0·49	0·77	1·36	2·48
0·45	1·35	0·78	1·48
0·42	1·63	0·50	0·90
0·27	1·98	0·15	0·26
0·00	2·13	0·00	0·00
Culture 3B. Succinate as the carbon source and ammonium as the nitrogen source. Ammonium limiting at a concentration of 7·50 mM. in the inflowing medium.			
0·54	0·20	3·76	7·14
0·50	1·43	2·53	4·24
0·44	2·36	1·60	3·26
0·37	3·26	0·70	1·16
0·26	3·59	0·37	0·38
0·17	3·59	0·37	0·18
0·00	3·96	0·00	0·10

* Values of R, P_e, and S_e are averages of several determinations.

† This term is an estimate of the concentration of the limiting nutrient in units of population density. The quantity cS_0 is constant for each culture and equal to value of P_e at R equal to zero.

‡ Estimates of this term were made by direct chemical analysis and are expressed in millimoles per l.

Monod (1942) showed that the relationship between the specific growth rate of a bacterial population (R) and the concentration of a limiting nutrient (S) is approximately represented by

$$R = \frac{R_m S}{A+S}. \tag{4}$$

Parameters R_m and A are, respectively, the maximum specific growth rate of the culture and the concentration of the limiting nutrient when the specific growth rate is $\frac{1}{2}R_m$. The same relationship was found by Schaefer (1948) and by Longmuir (1954).

At equilibrium in continuous cultures, from equations (3) and (4),

$$D = R = \frac{R_m S_e}{A+S_e}, \tag{5}$$

where S_e is the concentration of limiting nutrient at equilibrium (Monod, 1950; Herbert *et al.* 1956; Moser, 1958). It is apparent from equation (5) that as S_e approaches S_0 and, from equation (2), as P_e approaches zero, R approaches a critical value R_c (see Herbert *et al.* 1956), where

$$R_c = \frac{R_m S_0}{A+S_0}. \tag{6}$$

Estimates of R_c can be obtained for each culture from the intersection on the y-axis of the curve giving R as a function of P_e (Fig. 2). Of particular interest is the fact that the estimates of R_c are the same for each culture with the same nitrogen and carbon sources, irrespective of the kind or concentration of the limiting nutrient. Thus, from equation (6), the ratio of the values of A for each of two such cultures is equal to the ratio of their values of S_0, or

$$\frac{A'}{A''} = \frac{S_0'}{S_0''}.$$

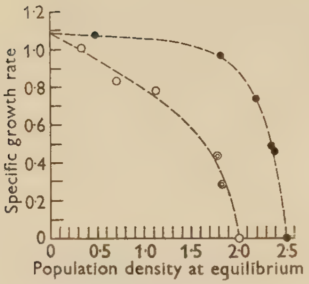

Fig. 2*a*

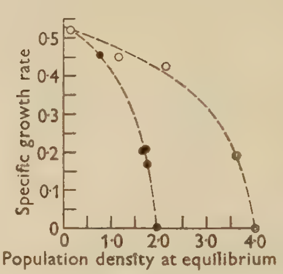

Fig. 2*b*

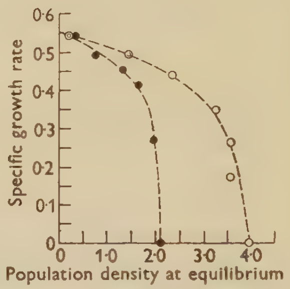

Fig. 2*c*

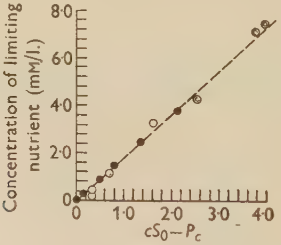

Fig. 3

Fig. 2*a*, *b* and *c*. Relationship between specific growth rate and population density of continuous cultures of *Aerobacter aerogenes* at equilibrium. (*a*) Data of Table 1; ● = culture 1A, glucose limiting at 5·55 mM. in inflowing medium; ○ = culture 1B, ammonium limiting at 3·75 mM. in inflowing medium, glucose as carbon source. (*b*) Data of Table 2; ● = culture 2A, succinate limiting at 8·47 mM. in inflowing medium; ○ = culture 2B, succinate limiting at 16·95 mM. in inflowing medium. (*c*) Data of Table 3; ● = culture 3A, ammonium limiting at 3·75 mM. in inflowing medium, succinate as carbon source; ○ = culture 3B, ammonium limiting at 7·50 mM. in inflowing medium, succinate as carbon source.

Fig. 3. Relationship between concentration of limiting nutrient (ammonium) and estimates of concentration of limiting nutrient in units of population density ($cS_0 - P_e$) in continuous cultures of *Aerobacter aerogenes* at equilibrium. Data of Table 3; ● = culture 3A, ○ = culture 3B. See text for definitions of terms.

Since S_0 can be set at will, A must be a function of S_0.

Further evidence of this relationship is obtained from consideration of the data of Tables 2 and 3. Rearrangement of equation (5) gives

$$\frac{1}{R} = \frac{1}{R_m} + \left(\frac{A}{R_m}\right)\left(\frac{1}{S_e}\right). \tag{7}$$

If A is a function of S_0, $1/R$ should be a linear function of $1/S_e$ for each culture, but with slope dependent upon S_0. Figure 4 shows regressions of $1/S_e$ upon $1/R$ for the data of Tables 2 and 3 fitted by the method of least squares. The slopes of the regression lines are, respectively, 3·66 and 5·90 for cultures 2A and 2B; and 0·47 and 0·72 for cultures 3A and 3B. These results show that the values of A are correlated with S_0 in each instance and can be considered to be proportional to S_0, or

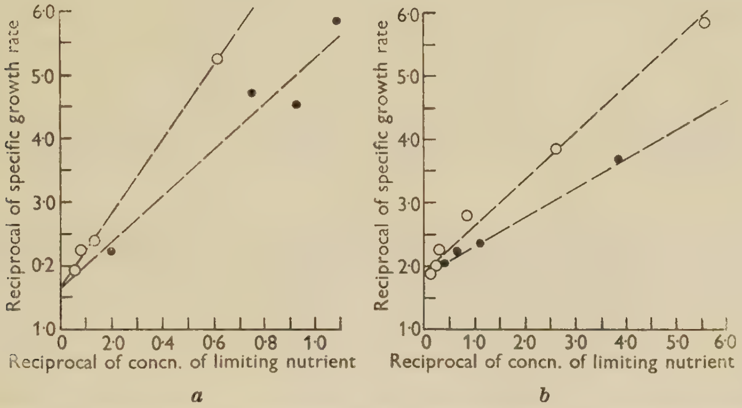

Fig. 4*a* and *b*. Regression of reciprocals of the concentration of limiting nutrient upon reciprocals of specific growth rate in continuous cultures of *Aerobacter aerogenes*. (*a*) Data of Table 2; ● = culture 2A, ○ = culture 2B. (*b*) Data of Table 3; ● = culture 3A, ○ = culture 3B.

$$A = bS_0. \tag{8}$$

Dr A. Novick (personal communication) has pointed out that $A = a + b'S_0$ is a more realistic relationship in that on conversion of A into terms of P_e and S_e by equation (2) and substitution into equation (4) a model is obtained that is better behaved at small values of P_e. However, the precision of the data is not good enough to determine whether or not a is real. Moreover, equation (8) is sufficient for the purposes of this discussion since a, even if real, would be quite small compared to the values of bS_0 in these experiments.

Substituting equation (8) into (7) gives, therefore,

$$\frac{1}{R} = \frac{1}{R_m} + \left(\frac{bS_0}{R_m}\right)\left(\frac{1}{S_e}\right) \tag{9}$$

and, from equation (2) and letting $(1+b)/R_m = 1/u_m$ and $b/cR_m = B/u_m$,

$$\frac{1}{R} = \frac{1}{u_m} + \left(\frac{B}{u_m}\right)\left(\frac{P_e}{S_e}\right). \tag{10}$$

Equation (10) requires that the reciprocal of the specific growth rate should be a linear function of the ratio of population density to concentration of limiting nutrient. Plots of $1/R$ versus P_e/S_e for the data of Tables 1–3 are given in Fig. 5, and confirm the relationship given by equation (10). The different slopes of Fig. 5*a* arise from the fact that the limiting nutrient is different in each culture.

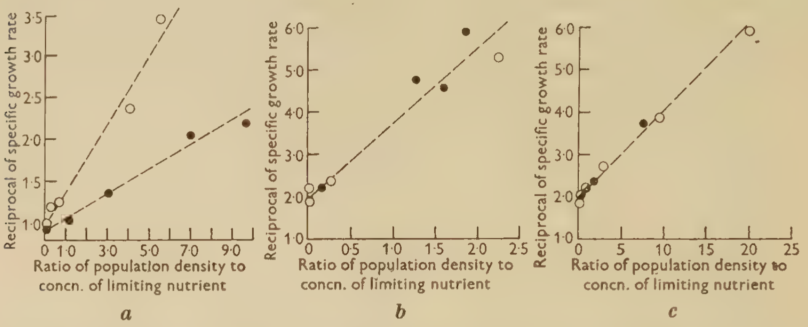

Fig. 5 *a*, *b* and *c*. Relationship between reciprocals of specific growth rate and ratios of population density to concentration of limiting nutrient in continuous cultures of *Aerobacter aerogenes* at equilibrium. (*a*) Data of Table 1; ● = culture 1A, ○ = culture 1B. (*b*) Data of Table 2; ● = culture 2A, ○ = culture 2B. (*c*) Data of Table 3; ● = culture 3A, ○ = culture 3B. ◒ indicates two points at the same locus.

Rearrangement of equation (9) gives

$$R = \frac{R_m S}{bS_0 + S} \tag{11}$$

and equation (10) gives

$$R = \frac{u_m S}{BP + S}. \tag{12}$$

DISCUSSION

Monod (1942), in deriving equation (4), and others, who subsequently used this relationship, considered parameter A to be independent of population density. Most of Monod's data, however, was obtained from experiments in which specific growth rates were measured at the beginning of growth cycles of batch cultures with different concentrations of limiting nutrient and in which initial population densities were the same from culture to culture. His results could be explained by equation (12) since, with population density essentially constant, equation (12) would reduce to equation (4). In studies of batch cultures where specific growth rates and concentrations of limiting nutrient were measured throughout the entire growth cycle, the relationship between specific growth rate and nutrient concentration for each culture could be expressed by equation (11); since, with initial concentration of limiting nutrient constant in each instance, equation (11) also reduces to equation (4).

Although the results of this study show that parameter A—the concentration of the limiting nutrient when the specific growth rate is half maximum—is a function of population density, the data provide no insight into the origin of or reason for this relationship. Other workers have attributed deviations of continuous cultures from Monod's model (equation (4)) to 'apparatus effects' such as incomplete or 'imperfect' mixing of the inflowing medium with the culture and wall-growth (Herbert *et al.* 1956). Although 'perfect' mixing is difficult to attain in continuous culture devices with large culture volumes, the small culture volumes (30–45 ml.) and the methods of mixing and introduction of medium used in these studies would preclude incomplete mixing as a factor responsible for the observed results. Further tests, which consisted of introducing drops of a dye solution into the culture vessels, showed that mixing was completed in only 2 or 3 sec. As previously mentioned, no wall-growth was observed in any of these experiments.

The point could be legitimately raised that the observed relationship between population density and parameter A might be due to consequential changes in environmental conditions as population density becomes large, or to the possibility that oxygen may become limiting at high growth rates (cf. Pirt, 1957). The author believes that these factors were not operative in these studies since the observed growth parameters for each culture were independent of population density, nutrient concentration and specific growth rate. Also, under comparable conditions they were reproducible from culture to culture independently of the concentration of the limiting nutrient in the inflowing medium. The results observed in these experiments might be attributable to an inhibition of the growth process by end-products of that process. Since the yield coefficient is independent of specific growth rate in each culture, the concentrations of soluble end-products of the growth process probably are related to P (or S_0) in a simple manner. Thus the appearance of P in equation (12) may be due to what is primarily an effect of concentration of end-products.

The nature of equation (11) (or (12)) is such that, discounting lag, so-called logarithmic growth could never occur in batch cultures. However, parameter b is small for most cultures; and, as a result, with population density small and concentration of limiting nutrient relatively large (conditions usually extant in 'young' batch cultures) growth could be considered logarithmic for all practical purposes over a considerable time interval. For example, an organism growing in batch culture with an initial population density of 1×10^6 organisms/ml., cS_0 equal to 2×10^9 organisms/ml., R_m equal to 0·5 hr.$^{-1}$, and b equal to 0·1 would have an initial specific growth rate (by equation (11)) of 0·454. After *c.* 10 hr. of growth and at a population density of 1×10^8 organisms/ml. the specific growth rate would still be 0·452.

In conclusion, it is believed that equations (11) and (12) serve as adequate models for bacterial growth in both batch and continuous cultures and are more realistic than other models proposed previously. By substitution of equation (12) into equation (3) and integrating with dilution rate constant, a relationship is obtained which shows that population density and concentration of limiting nutrient approach constant values in time which are unique for each dilution rate less than the maximum specific growth rate. This accounts for the relationship between specific growth rate and population density at equilibrium that has been observed in this and other studies (see Herbert *et al.* 1956) and the fact that equilibrium is even attained by continuous cultures.

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Mode of Action of Megacin

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SUMMARY: Megacin however highly bactericidal for sensitive organisms was not adsorbed by them. Its bactericidal action was markedly dependent on temperature. The viability of organisms exposed to megacin at 0° was not affected. When megacin was added to exponentially growing *Bacillus megaterium* there was cessation of growth followed by a gradual decrease in turbidity of the culture. The decrease in turbidity was, however, not associated with a total lysis of individual organisms; rather it was the consequence of the escape of the dense intracellular material from the organisms. Intracellular components, i.e. substances which absorbed in the ultraviolet region escaped from the bacteria into the medium while cell wall remained essentially intact. When *B. megaterium* suspended in a medium containing lactose was exposed to megacin, β-galactosidase appeared on the surface of bacteria.

Protoplast preparations made from *Bacillus megaterium* and *Micrococcus aurantiacus* (both sensitive to megacin) were converted into ghost-like structures on the addition of megacin. On the other hand, protoplasts made from insensitive species resisted megacin. Observations indicate that megacin causes a radical change in the osmotic barrier of sensitive organisms by attacking the cytoplasmic membrane. Data available suggest that megacin is either an enzyme which breaks down the osmotic barrier of sensitive cells, or is a substance capable of activating the intrinsic enzymes of cells which lead to an autolysis of cytoplasm.

Knowledge of the mode of action of megacin, an antibacterial principle obtained from *Bacillus megaterium* (Ivánovics & Alföldi, 1954), is scanty. In preliminary investigations, lysates of megacinogenic bacteria obtained in complex media were shown to exert a bactericidal action on organisms of sensitive strains. Furthermore, the addition of these lysates to cultures of *B. megaterium* growing exponentially resulted in a rapid cessation of the oxygen uptake (Ivánovics & Alföldi, 1955). In order to study the specificity of megacin action lysates containing megacin were partially purified and used instead of crude lysates. The purification of megacin has been made easier by the fact that the synthesis of megacin will take place in organisms growing in defined medium in the presence of an adequate concentration of manganese (Alföldi, 1957). Lysates produced in defined culture medium served for obtaining megacin concentrates of high potency. Experiments carried out with these concentrates give some information regarding the mode of action of this particular bacteriocin.

METHODS

Organisms. Bacillus megaterium, strain 216, and the phage resistant 'Mut-C' strain were the same as those described previously (Ivánovics & Alföldi, 1955; Ivánovics, Alföldi & Széll, 1957). Strain KM came from the laboratory of Dr C. Weibull (Central Bacteriological Laboratory of Stockholm City). The other strains used in these investigations were from the collection of this Institute.

Culture media. Horse meat-extract agar containing 1 % (w/v) peptone and 1·5 % (w/v) agar. The defined culture medium was prepared as described by Alföldi (1957). Our previous work provides information concerning other culture media and methods (Ivánovics & Alföldi, 1955, 1957).

The titration and unit of megacin. One ml. of the young culture of the indicator strain 'Mut-C' (0·1 unit optical density equivalent to 0·07 mg. dry wt. organism/ml.) mixed with 1 ml. melted meat-extract agar was layered on agar plates. From the material containing megacin twofold serial dilutions were prepared and, by the aid of a platinum loop, 0·02 ml. amounts were placed on the plates, which were then incubated overnight at 37°. For more accurate titrations we prepared dilutions at closer intervals. In principle the highest dilution of which 0·02 ml. produced inhibition represents the unit of megacin. For the sake of convenience not this, but the fifty-fold amount, was taken as the unit, i.e. the reciprocal value of the titre expresses the number of megacin units/ml. When new lots of lysate or concentrate were assayed a concentrate of known activity was also included in the test.

Megacin concentrates. Megacin concentrates were obtained from *Bacillus megaterium*, strain 216, growing in a defined medium (Alföldi, 1957). The method of preparing megacin concentrates is outlined below. Two hundred ml. of defined culture medium in a 1 l. Erlenmeyer flask was inoculated with *B. megaterium*, strain 216. The inoculum was made from an overnight culture on a slope or YDC agar, the growth being suspended in 5 ml. saline; 1 ml. of this was used to inoculate 200 ml. medium. Cultivation was carried out with vigorous shaking (180 shakes/min., 4 cm. in amplitude) at 37°. When the culture attained an optical density of 0·5–0·6 unit (equiv. 0·55–0·75 mg. dry wt. organism/ml.), some of it was poured into a flat enamelled dish so that the layer of liquid was 2 mm. thick. The liquid was kept in violent motion and irradiated with a low-pressure ultraviolet lamp (230 erg./mm.^{2}). The irradiated culture was transferred to a flask and re-incubated for another 4 hr. under the same conditions as before. During this period there was first some growth, followed by partial lysis. At the end of the incubation period the pH value of the culture was adjusted with $0·07\,\text{M-Na}_2\text{HPO}_4$ to pH 7·2 and the culture kept in an ice-box till the next day. The supernatant fluid obtained after centrifugation usually contained 40,000 units megacin/ml. To every 100 ml. of the solution we then added 30 ml. of 96 % (w/v) ethanol in water, followed by dialysis for 2 days in cellophan sacs against distilled water at 4°. The ethanol suppressed the growth of possible contaminants.

From the solution of low electrolyte content thus prepared, the active principle was readily adsorbed by the anion-exchange cellulose Ecteola (Peterson & Sober, 1956), while no adsorption took place on acid-cellulose CM-W (Peterson & Sober, 1956). Ecteola (2·5 g.) was added to 500 ml. dialysed megacin solution and the suspension was stirred for 1 hr. at room temperature. After centrifugation the supernatant fluid was carefully poured off and the sediment resuspended in 50 ml. buffered 1 % (w/v) NaCl. The pH value of the saline was adjusted to pH 7 with phosphate buffer 0·07M; 5 ml. buffer/100 ml. saline. Elution was carried out by stirring at room temperature for 1 hr. The liquid containing the megacin was separated from the cellulose preparation by centrifugation.

Depending on the starting materials, etc., in different experiments the eluates contained 200,000 to 300,000 units megacin/ml. In six attempts at concentration the yield was 40–56 % of the original megacin. The nitrogen content of the concentrates varied between 50 and 90 μg. N/ml. Since the nitrogen content of dialysed lysates in individual lots was *c.* 60 μg. N/ml. thus roughly fivefold purification was achieved during concentration. After being sterilized by passage through sintered glass bacterial filters the concentrates could be kept unchanged for several months in an ice-box. The concentrates did not contain proteolytic enzymes; no effect on gelatin films observed even after prolonged standing.

Preparation of protoplasts. Bacillus megaterium was grown from a heavy inoculum from an overnight agar slope in a defined medium (Alföldi, 1957) with vigorous shaking for 6–7 hr. at 37°. Cultures containing equiv. 1–1·5 mg. dry wt. organism/ml. were centrifuged and the bacteria washed twice with buffered saline. The bacteria were then resuspended in medium PM which contained 0·02 M-phosphate buffer (pH 7·2) + 0·2 M sucrose + 0·01 M-$MgSO_4.7H_2O$. Crystalline lysozyme was added (100 μg./ml.) to the suspension which was then incubated at 37°. A stable protoplast suspension was obtained within a few minutes. Protoplasts were prepared in a similar manner from *Sarcina flava, Micrococcus aurantiacus* and *B. subtilis*. These organisms were harvested from a horse-meat peptone broth after growth at 37° for 8 hr. In the case of *B. subtilis*, a lysozyme concentration of 300 μg./ml. was used for making the protoplasts. From Gram-negative bacteria only penicillin-induced 'protoplasts' were studied, prepared according to the technique of Lederberg (1956).

Buffered saline solution. 0·9 g. NaCl/100 ml. distilled water + 5 ml. 0·07 M-phosphate buffer (pH 7·2).

Assay of β-galactosidase. This was done by the method of Landman (1957) with *o*-nitrophenyl-β-D-galactoside.

Crystalline ribonuclease. This was prepared after McDonald (1955) and kindly given by Professor B. F. Straub (Budapest).

Lysozyme was prepared from egg white by isoelectric point crystallization.

Trypsin ('1:250') was bought from Difco Laboratories, U.S.A.

Polymyxin B sulphate. (Chas. Pfizer and Co. Inc. U.S.A.); 1 mg. equivalent to 10,000 units was used in these experiments.

RESULTS

Adsorption and bactericidal effect of megacin

Some of the known antibiotics of polypeptide character such as polymyxin B are highly surface-active and are therefore readily adsorbed in a non-specific manner by bacteria. Their bactericidal effect (due to surface-activity; Bliss, Chander & Schoenbach, 1949) is only slightly influenced by temperature. With this in mind it seemed of interest to do similar experiments with megacin concentrates. One of our megacin concentrates (4000 units/μg. N) had exactly the same stalagmometric value as the solvent alone. On the other hand, polymyxin B (100 μg./ml.) markedly decreased the surface tension of saline. Further, megacin was not significantly adsorbed by a thick suspension (equiv. 10 mg. dry wt. organism/ml.) of megacin-sensitive *Bacillus megaterium* KM.

Suspensions of *Bacillus megaterium* were exposed to megacin at different temperatures, and samples taken at intervals for plating to determine the colony count. It can be seen from Fig. 1 that whereas the viability of organisms exposed to megacin at 0° was not affected, there was a progressive decrease in viability with increase in temperature and with increase in time of exposure to megacin.

Decrease of turbidity of suspensions by the action of megacin

When megacin was added to exponentially growing cultures of *Bacillus megaterium* shaken at 37°, their turbidity gradually decreased. The results of a typical experiment carried out with strain KM is shown in Fig. 2. It can be

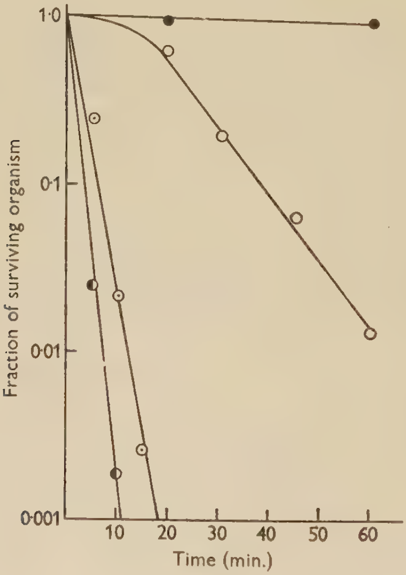

Fig. 1

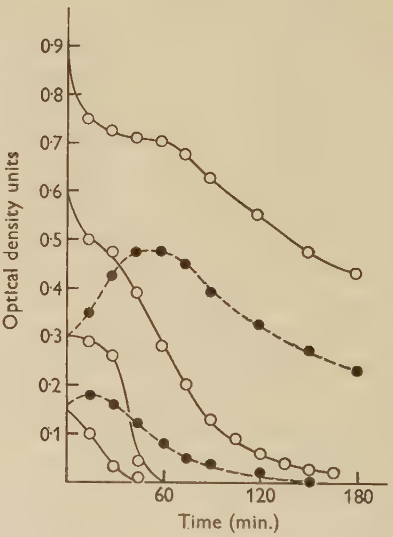

Fig. 2

Fig. 1. Bactericidal effect of megacin at different temperatures. A young broth culture of *Bacillus megaterium*, strain KM, was distributed into test tubes previously brought to the required temperature. Megacin was added to give a final concentration of 200 units/ml. Initially the number of colony formers was $1{\cdot}55 \times 10^6$/ml. At intervals appropriate dilutions were made with ice-cold broth and 0·1 ml. of suitable dilutions plated on meat extract agar. ●, at 0°; ○, at 12°; ⊙, at 29°; ◐, at 40°.

Fig. 2. Effect of megacin on turbidity of cultures of *Bacillus megaterium*, strain KM, grown in meat-extract broth or in defined medium. Megacin (2000 units/ml.) was added to the cultures which were then re-incubated with gentle shaking at 37°. ● = in defined medium; ○ = in meat-extract broth.

seen that the addition of megacin to cultures in meat-extract broth led to a rapid cessation of growth, while with the cultures in the defined medium there was some growth before the decrease in turbidity. The rate of decrease in turbidity was highly dependent on the medium used and also on the initial optical density, i.e. the age of the culture. Similar observations were made with strain 'Mut-C', a phage-resistant mutant of *B. megaterium*. However, in this case the decrease in turbidity of cultures was less marked. When young cultures of *B. megaterium* strains were mixed with megacin and then poured into test tubes so that there was a deep layer of culture and these were incubated statically and thus under semi-anaerobic conditions, the results were variable. Sometimes lysis occurred in the control broth cultures in the absence of added megacin while, on the other hand, megacin did not always cause a decrease in the turbidity of cultures grown in a defined medium.

The addition of megacin to killed bacterial cultures of *Bacillus megaterium* did not cause a decrease in turbidity. The turbidity of heat-killed organisms (60° for 30 min.) and the turbidity of cultures sterilized by chloroform remained unaltered after adding high concentrations of megacin. Megacin concentrates did not exhibit a clearing effect when spotted on the surface of a soft agar layer containing a suspension of killed *B. megaterium* KM. This technique, used by Murphy (1958) for assaying a phage-associated enzyme of *B. megaterium*, did not reveal any lytic action of megacin concentrate on the killed organisms.

Cultures of *Bacillus megaterium* exposed to megacin were examined at intervals under oil immersion in a phase-contrast microscope. Before the addition of megacin, the cytoplasm of the organisms was very dense and showed a dark greyish violet structure. As the time of exposure to megacin increased, the homogeneity of the cytoplasm gradually decreased and distinct granules appeared in the interior. This change was associated with shrinkage of the contour of the organism. Finally, when a very marked decrease in the optical density of the culture had taken place, the organisms either seemed empty or only large dark granules were seen in their interior.

When counts were made in a haemocytometer the number of empty organisms was found to be identical with the number of organisms present at the time of addition of megacin to the culture (Table 1). This experiment indicated that the change in turbidity of the culture under the action of megacin was not due to a total lysis of the organisms but rather to the escape of optically dense intracellular materials from the organisms. This leakage of cell contents was confirmed by taking samples at intervals, removing solid materials by centrifugation and then measuring the absorption of the supernatant fluids at 260 mμ. It can be seen from Fig. 3 that there was a steady increase in the u.v.-absorbing capacity of the supernatant fluids. An escape of cell material was also observed when megacin was present in a concentration so dilute that there was no marked decrease of turbidity within a short time. This was made apparent by assays of the adaptive enzyme β-galactosidase in suspensions harvested from media containing lactose instead of glucose. Megacin appeared to disrupt the osmotic barrier in bacteria and thus allow the *o*-nitrophenyl-*β*-D-galactoside to reach the enzyme. In the control suspension (without megacin) the value of *β*-galactosidase remained permanently very low during the whole period of the experiment and served as a blank in photometric estimates of the *o*-nitrophenol split by the *β*-galactosidase.

Microscopic and chemical investigations of cultures containing megacin suggested that there was a marked change in the osmotic barrier of organisms. The decrease in turbidity of the culture was, however, only an apparent lysis.

Table 1. *The turbidity and haemocytometer count of* Bacillus megaterium, *strain KM, culture in broth after the addition of* 2000 *units megacin/ml.*

Time (min.)	Optical density units	Count/ml.*
0	0·120	$1·97 \times 10^7$
20	0·100	$2·16 \times 10^7$
50	0·000	$1·00 \times 10^7$

* 1/10 dilutions of samples were made with saline containing 0·01 % crystal violet + 0·1 % formalin and counted in a Buerker chamber.

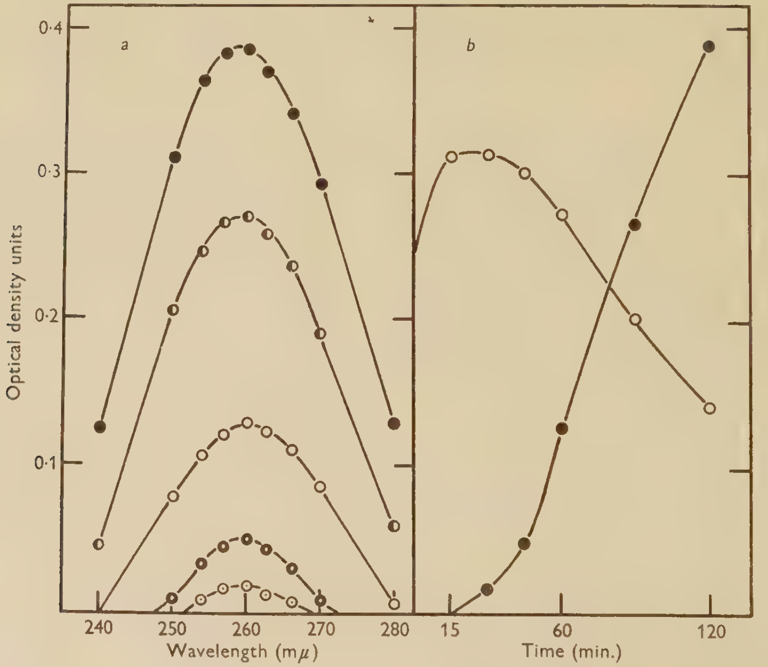

Fig. 3. Escape of u.v.-absorbing material from *Bacillus megaterium*, strain KM, in the presence of megacin. Megacin (2000 units/ml.) was added to a culture growing exponentially in defined medium and re-incubated, with gentle shaking, at 37°. Samples were taken at intervals, cooled and centrifuged. The u.v.-absorbing capacity of supernatant fluids was measured between 240 and 280 mμ. (*a*) Absorption curves of individual samples; ⊙, 30 min.; **O**, 45 min.; ○, 60 min.; ◐, 90 min.; ●, 120 min. (*b*) ○ = optical density values of suspension at 590 mμ; ● = extracellular fluid at 260 mμ at different times.

In fact, the cell wall was left uninjured or only slightly injured, indicating that the primary action of megacin was exerted not on the cell wall itself but on deeper layers of the organisms. This increased the permeability of the osmotic barrier.

Megacin action on protoplasts

Very stable preparations of protoplasts were made by lysozyme treatment of strains KM and 'Mut-C' of *Bacillus megaterium*. The optical densities of preparations were measured in the presence of various concentrations of megacin (Fig. 5). By microscopic observation, it was found that the presence of megacin caused the transformation of protoplasts into 'ghosts'; the latter appeared before there was a significant change in the optical density of the suspension. This result again supported the view that the megacin acted on the osmotic barrier of the cells.

There is now good evidence which indicates that the action of megacin concentrate is specific and due to its bacteriocin content. Autolysates as well

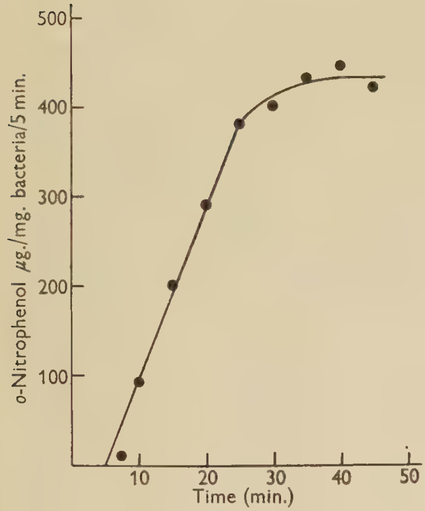

Fig. 4

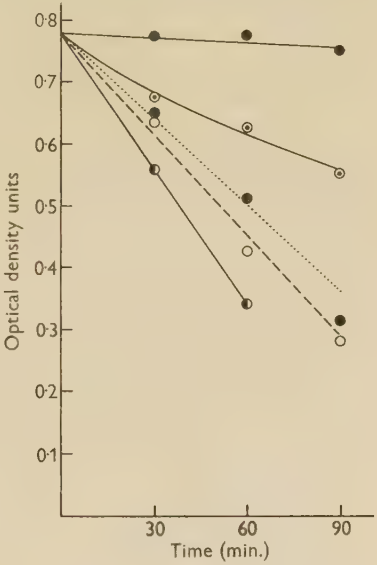

Fig. 5

Fig. 4. The appearance of β-galactosidase in suspensions of *Bacillus megaterium*, strain KM, in the presence of megacin. Organisms were grown in defined medium but with glucose replaced by lactose, collected by centrifugation and washed twice with saline. The washed organisms were resuspended to equiv. 1 mg. dry wt./ml. Megacin (200 units/ml.) was added to suspensions preheated to 37° and kept at this temperature. At intervals, 0·1 ml. samples were taken and added to 2 ml. *o*-Nitrophenyl-β-D-galactoside-containing assay medium. After 5 min. β-galactosidase activity was stopped by adding 2 ml. ethanol (96 %, w/v). The intensity of colour developed in the cell-free fluid was estimated at 420 mμ in a Beckman photometer.

Fig. 5. The change in optical density of protoplast suspensions prepared from *Bacillus megaterium*, strain KM, in the presence of various concentrations of megacin. Megacin was added to the protoplast suspension pre-heated and incubated at 37°. ●—, Control; ⊙—, 400 units megacin/ml.; ●····, 2×10^3 units/ml.; ○----, 4×10^3 units/ml.; ◐—, 1×10^4 units/ml.

as extracts obtained by lysozyme treatment from washed non-induced organisms of megacinogenic strain 216 of *Bacillus megaterium* did not transform protoplasts into ghosts. On the other hand, when cultures of strain 216 were u.v.-irradiated and re-incubated, the appearance of the principle responsible for protoplast destruction paralleled the increase in the antibacterial action of the cell extract, i.e. the elaboration of megacin. The megacin concentrates used in the above experiments were devoid of any proteolytic activity. Our protoplast preparations resisted treatment with trypsin ('1:250') at 100 μg./ml. No change in the structure of protoplasts was seen under the microscope following the addition of crystalline ribonuclease (100 μg./ml.) or a megaterium phage suspension of high potency.

The specificity of megacin action

Megacin has a very narrow antibacterial spectrum which includes only strains of *Bacillus megaterium* and some coloured cocci. A small proportion of strains of *B. anthracis* and B. *subtilis* tested appeared to be slightly sensitive to crude lysates of megacinogenic strains (Ivánovics, Alföldi & Abraham, 1955). Whether this latter action can be considered specific or not needs further investigation. Megacin concentrates of high activity now offer an opportunity to investigate the specificity of megacin action on protoplast preparations made from different organisms (Table 2). Some of our 'protoplast' preparations were made by the penicillin technique (Lederberg, 1956). While we designate all our preparations as 'protoplast', that is only for the sake of simplicity, since we accept the distinction between penicillin- and lysozyme-formed 'protoplasts' (Brenner *et al.* 1958). For comparison, a solution of polymyxin B was also included in these experiments.

Table 2. *Conversion of 'protoplasts' into ghosts by megacin or polymyxin B*

To suspensions of 'protoplasts', megacin (400–2000 units/ml.) or polymyxin B (100 μg./ml.) was added and incubated at 37° for 30 min.

		Conversion into ghost by	
Organism	Sensitivity to megacin	Megacin (400–2000 units/ml.)	Polymyxin B (100 μg./ml.)
Bacillus megaterium, strain KM	+	+	+
B. megaterium, strain Mut-C	+	+	+
B. megaterium, strain 216	+	+	+
Micrococcus aurantiacus	+	+	+
Bacillus subtilis, 302	–	–	–
B. subtilis, 304	–	–	–
Sarcina flava	–	–	–
Escherichia coli B*	–	–	+
Salmonella typhosa O*	–	–	+
*S. abony**	–	–	+
*Shigella schmitzii**	–	–	+

* Penicillin-induced spherical bodies.

Protoplasts made from strains of species sensitive to the bactericidal action of megacin were converted, without exception, into ghosts by this bacteriocin. Polymyxin B also effected the destruction of protoplasts of these strains and its action included the spherical bodies ('protoplasts') of Gram-negative bacteria. It is interesting to note that protoplasts of *Bacillus subtilis* and of *Sarcina flava* resisted polymyxin B and megacin.

In a quantitative experiment with protoplasts of *Bacillus megaterium*, strain KM, the lowest concentration of polymyxin B which brought about conversion of protoplasts into ghosts was 50–100 μg. (500–1000 units/ml.) while the effective limiting concentration of megacin was 200 units/ml. The megacin concentrate used in this experiment contained 2000 units megacin/μg. N.

DISCUSSION

The experiments described above indicate that megacin caused a radical change in the osmotic barrier of sensitive cells. Escape of intracellular components into the medium was a consequence of megacin action either on cells or on protoplasts of sensitive organisms. As this effect was not observed on heat- or chloroform-killed organisms this suggests a primary action on the function of the osmotic barrier, followed by the death of sensitive organisms. The fact that megacin did not lyse killed organisms, nor attack the cell wall of living ones distinguishes its action from that of the enzyme associated with megaterium phage (Murphy, 1958) which dissolves the cell wall of *Bacillus megaterium*. The available evidence does not allow more precise definition of the actual point of attack of megacin.

The very narrow antibacterial range of megacin (Ivánovics, Alföldi & Abraham, 1955) points to a highly specific action of megacin on sensitive organisms. The specificity of antibacterial action is in a good accordance with the results of experiments made with protoplasts. Only protoplasts prepared from *Bacillus megaterium* and *Micrococcus aurantiacus* were attacked by megacin; those of *Sarcina flava* and *B. subtilis*, which are not sensitive to megacin, were spared. Although 'spherical bodies' of Gram-negative bacteria induced by penicillin cannot be considered as true protoplasts (Brenner *et al.* 1958) they give ghost-like structures when treated with polymyxin; megacin apparently left these spheres untouched. The action of megacin is reminiscent of the enzyme plakin (Amano, Kato & Shimizu, 1952) isolated from horse platelets. Plakin converts protoplasts of *B. megaterium* into ghosts (Amano *et al.* 1956). It should be stressed, however, that its action is not so highly specific as that of megacin: its antibacterial action and its action in converting protoplasts into ghosts is not confined to *B. megaterium* but affects several of the genus *Bacillus*.

We have failed so far to establish the enzymic character of megacin by enzyme kinetic studies. That its bactericidal action is highly dependent on temperature cannot be considered as evidence for a primary enzyme function, for this would also be the case if an autolytic process in cells or protoplasts were enhanced by megacin. One may wonder about the biological significance of the production of this particular bacteriocin. Megacinogeny is reminiscent of lysogeny except for the liberation of mature phage particles or the production of defective phage (Ivánovics, Alföldi & Lovas, 1957). This question has already been discussed (Ivánovics & Alföldi, 1957) and we cannot exclude a possible synthesis of non-organized phage material governed by a highly degraded prophage. Jacob & Fuerst (1958) described the enzyme endolysin, elaborated by mutants of lysogenic *Escherichia coli* K12 which were made highly defective artificially. Endolysin is claimed to be an enzyme which attacks cell-wall constituents of several Gram-negative bacteria. So far, we have failed to obtain from typically lysogenic strains of *Bacillus megaterium* defective mutants capable of producing megacin instead of phage particles.

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On the Nature of the Selective Action of Selenite Broth

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SUMMARY: Sodium selenite is toxic in low concentration to salmonellas as well as to other enterobacteria. The ability of salmonellas to grow in selenite broth appears to be due to binding of selenite by constituents of peptone (e.g. peptides). Reduction of selenite takes place after growth is established and the intensity of reduction is related to the profuseness of growth. The presence of a fermentable carbohydrate favours growth and intensity of selenite reduction. Many organisms, however, die as a result of the reducing conditions. Organisms differ in their ability to use different sulphur sources; there was evidence that cystine and pentathionate as highly preferred sulphur sources may be of special significance. Sodium selenite reacts with certain inorganic sulphur compounds to form seleno-polythionates. It is believed that the selectivity of selenite broth may be due to the presence of seleno-polythionates or other seleno-sulphur compounds which act as growth-inhibitory analogues of highly preferred sulphur sources.

That bacteria are capable of reducing selenium salts to selenium has been known for over a century and has been the subject of a number of investigations. A review of the literature was made by Levine (1925), who studied the effects of various selenium compounds upon growth and upon the reducing powers of a number of bacteria, and found that the compounds could be arranged in a series of diminishing toxicity, e.g. selenium dioxide (selenious acid), selenic acid, sodium selenite, sodium selenate, potassium selenocyanide. In cultures containing selenite or selenate there was formation of brick-red selenium which was partly precipitated and partly colloidal. Reduction took place only when growth occurred and the degree of reduction paralleled growth, an observation also made by Klett (1900). With diphtheria bacilli, Levine found granules of selenium deposited within the cells and regarded reduction as an intracellular phenomenon.

Leifson (1936) produced an enrichment medium containing sodium selenite ($NaHSeO_3$) which is now widely used for the routine isolation of salmonellas. Little is known about the reasons for the selectivity of this medium. Selenite broth is a relatively simple medium and, as stressed by Leifson, contains no chemicals of unknown composition except the peptone. It appeared to be a suitable medium for the present study, which is concerned with an attempt to elucidate the basis of selectivity of selenite broth. Because of the emphasis laid on the reduction of selenium compounds by bacteria it seemed important to study the growth of different enterobacteria in selenite broth and the significance of the reduction of the selenite in relation to the selectivity of the medium. It was felt that little headway would be made without more knowledge of the nutrition of the organisms used. From this aspect a number of strains of *Salmonella choleraesuis* have been included among the organisms studied since this serotype is inhibited in selenite broth (Williams Smith, 1952) and any differences between it and *S. typhimurium* which might be revealed might help to elucidate the nature of the selective action of selenite broth.

METHODS

Media. The formulae for the different culture media used are shown in Table 1. For peptone, Difco Proteose–Peptone was used throughout. The media were as follows.

Selenite broth. This was prepared according to Hobbs & Allison's modification (1945) of Leifson's formula (1936), in which mannitol is substituted for lactose. The medium was sterilized by steaming for 30 min.

Table 1. *Composition of media used*

	Selenite broth	Mannitol peptone medium	Depleted medium	Defined medium	Defined 'sulphur-free' medium
Peptone (g.)	5	5	5	—	—
Na_2HPO_4 (g.)	10	10	10	—	—
$(NH_4)_2HPO_4 2H_2O$ (g.)	—	—	—	4	4
KH_2PO_4 (g.)	—	—	—	0·4	0·4
$MgSO_4 7H_2O$ (g.)	—	—	—	0·5	—
NaCl (g.)	—	—	—	1	1
$MgCL_2 6H_2O$ (g.)	—	—	—	—	0·5
$NaHSeO_3$ (g.)	4	—	—	—	—
Carbohydrate (g.)	4	4	4	5	5
Charcoal (g.)	—	—	40	—	—
Distilled H_2O (ml.)	1000	1000	1000	1000	1000
pH	7·1	7·1	7·1	7·1	7·1

Mannitol peptone medium. This was prepared in the same way as selenite broth except that the sodium selenite was omitted and sterilization was carried out by autoclaving at 115° for 10 min.

Depleted medium. Mannitol peptone medium was adsorbed with 4 % (w/v) charcoal (Farnol 12 × S carbon). This produced a biuret-negative medium which was sterilized by autoclaving at 115° for 10 min.

Chemically defined medium. The defined medium shown in Table 1 is a modification of the medium described by Gale (1947). For comparison with other media mannitol was used as carbon source. Sterilization was by autoclaving at 115° for 10 min. The concentration of thiamine (hydrochloride) used was 25 μg./ml. and of L-cystine, L-cysteine and DL-methionine 20 μg./ml. Other amino acids were added to a final concentration of 100 μg./ml. Inorganic sulphur compounds used as supplements were added to a final concentration of 0·1 % (w/v).

'Sulphur-free' defined medium was also used for a small number of experiments.

Organisms. A number of strains of *Salmonella choleraesuis* was obtained from the National Collection of Type Cultures; other organisms were isolated from routine laboratory specimens. They included: serotypes of salmonellas, *Escherichia coli* I, *Citrobacter freundii* I, *Klebsiella aerogenes* I and *Proteus vulgaris*.

RESULTS

Growth of organisms of Enterobacteriaceae in selenite broth

Different types of organisms were selected for special study of their growth in selenite broth; colony counts were made by the method of Miles & Misra (1938). An average of two samples was taken for estimation of viable counts. Figure 1 shows the growth of *Salmonella typhimurium*, *Citrobacter freundii* I, *Proteus vulgaris*, *Escherichia coli* I and *Klebsiella aerogenes* I, from which it may be seen that growth of *E. coli* I and *K. aerogenes* I was suppressed in comparison with the other organisms. There was no evidence of reduction of selenite to selenium until after 8 hr. of incubation, when the selenite at first became yellowish and gradually changed to a brick-red colour. Reduction of selenite to selenium followed bacterial growth therefore, and took place when maximal growth was reached. At the same time that macroscopic reduction was observed selenium granules were also seen microscopically inside the organisms or adhering to their surfaces, with numerous selenium granules free in the medium.

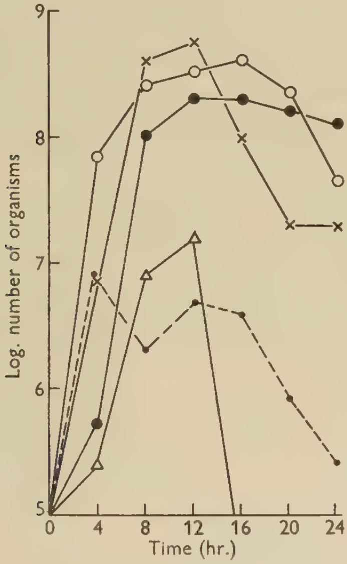

Fig. 1

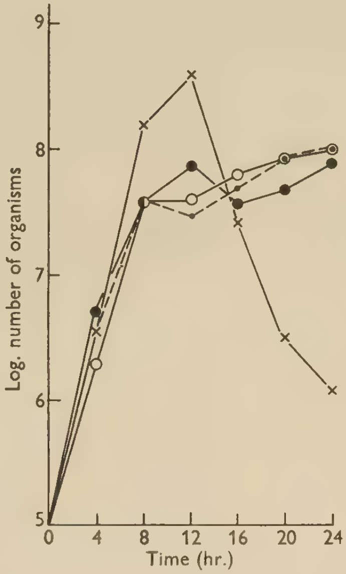

Fig. 2

Fig. 1. Growth of organisms in selenite broth. Inoculum 0·02 ml. × 10^{-1} of an overnight broth culture. —×—, *Salmonella typhimurium*; —○—, *Citrobacter freundii* I; —●—, *Proteus vulgaris*; —△—, *Escherichia coli* I; – – –, *Klebsiella aerogenes* I.

Fig. 2. Growth of *Salmonella typhi* in selenite broth containing different carbohydrates. Inoculum 0·02 ml. × 10^{-1} of an overnight broth culture. —○—, Lactose; —×—, mannitol; —●—, sucrose; – – –, no carbohydrate.

It was evident that the selective action of selenite broth was not dependent on reduction of sodium selenite and that it occurred early in the growth period, before reduction of selenite took place. Reduction of the selenium compound to selenium was extracellular and intracellular, and the intensity of reduction was proportional to the luxuriance of growth of the organisms. Growth was greatly enhanced, however, by the presence of a fermentable carbohydrate. This is illustrated by the growth of *Salmonella typhi* shown in Fig. 2. The more rapid decrease in colony count in mannitol selenite broth was accompanied by a much greater intensity of reduction of the selenium compound than in selenite broth without added fermentable carbohydrate.

The protective action of peptone on the toxicity of selenite

When peptone medium was treated with charcoal (Farnol 12 × S carbon) peptides were adsorbed and the medium became biuret-negative but still supported the growth of *Salmonella typhimurium.* Selenite broth treated in the same way with charcoal inhibited the growth of *S. typhimurium.* The inhibitory effect of charcoal on selenite broth emphasizes the importance of the peptone. In Table 2 the effect is shown of different concentrations of selenite in mannitol peptone medium. Though a concentration of 1 % (w/v) sodium selenite suppressed the growth of almost all the organisms tested, 0·5 % (w/v) allowed a good growth of some and was, therefore, a selective concentration. Comparison with different concentrations of selenite in depleted medium (Table 2) shows that in the biuret-negative medium selenite was toxic in low concentration to all the organisms tested. In depleted medium without selenite there was no growth of *S. typhi* or the laboratory strain of *S. choleraesuis* within 24 hr. Thus, the evidence from these experiments suggests that substances present in peptone, absorbable by the charcoal used, were necessary not only to protect organisms from the toxic effects of selenite but also to confer a degree of selectivity on the medium.

Table 2. *Growth of organisms in mannitol peptone medium with different concentrations of selenite*

Inoculum 0·02 ml. × 10^{-2} of an overnight broth culture to 10 ml. of the medium. Incubated at 37° for 24 hr. Heavy growth, + + + +; good growth, + + +; moderate growth, + +; slight growth, +; very slight growth, ±; no growth, –.

	Mannitol peptone medium					Depleted medium			
	Concentration of $NaHSeO_3$ % (w/v)								
	1	0·5	0·1	0·01	0	0·05	0·01	0·005	0
S. typhimurium	–	+ + +	+ + + +	+ + + +	+ + + +	–	±	+	+ + + +
S. typhi	–	+ + +	+ + +	+ + + +	+ + + +	–	–	–	–
S. choleraesuis	–	–	±	+ + +	+ + + +	–	–	–	–
E. coli I	–	±	±	+ +	+ + + +	–	±	+ +	+ + + +
P. vulgaris	±	+	+ +	+ +	+ +	–	±	±	+ +
C. freundii 1	–	+ + + +	+ + + +	+ + + +	+ + + +	–	±	+ +	+ + + +

Comparison of enterobacteria in different media

The importance of protein derivatives such as peptides in relation to toxicity provides some grounds for suspecting that the basis of selectivity of selenite broth is largely nutritional. The growth characteristics, therefore, of a number of organisms were compared in selenite broth, mannitol peptone medium, depleted medium and defined medium. In Table 3 it may be seen that the growth of *Escherichia coli* I, though suppressed in selenite broth, was luxuriant in the three non-selective media. On the other hand, *Citrobacter freundii* I was able to grow well in all the media except defined medium; in defined medium its growth was largely dependent on the size of the inoculum. Growth of all the strains of *Salmonella choleraesuis* was inhibited in selenite broth; three strains did not grow in depleted medium but produced a good growth in 24 hr. when thiamine was added; defined medium, even with the addition of thiamine, was inadequate for growth of all strains.

Table 3. *Growth of organisms in different media*

Inoculum 0·02 ml. × 10^{-3} of an overnight broth culture. Incubated at 37° for 24 hr. Arbitrary assessment of growth as before.

	Selenite broth	Mannitol peptone medium	Depleted medium	Defined medium
S. typhimurium	+++	++++	++++	++++
E. coli I	±	++++	++++	++++
C. freundii I	+++	++++	++++	–
S. choleraesuis strains				
Lab. strain	–	++++	–	–
NCTC 5364	–	++++	–	–
NCTC 5735	–	++++	–	–
NCTC 6678	–	++++	++	–
NCTC 5736	–	++++	++	–
NCTC 5737	–	++++	++	–
NCTC 5738	–	++++	++	±

Hydrogen sulphide production and selenite reduction

Observations of growth in selenite broth showed that intense reduction of selenite to selenium did not take place until the peak of the logarithmic phase had been reached. This was roughly similar in time to the production of H_2S as indicated by blackening of lead acetate paper strips. H_2S is known to react with selenious acid according to the equation

$$H_2SeO_3 + 2H_2S = 2S + Se + 3H_2O.$$

When Na_2S is added to $NaHSeO_3$ in solution, sulphur is first precipitated, followed by precipitation of selenium. H_2S production cannot be detected with lead acetate paper when using selenite broth; this is understandable in view of the reaction with selenite. The activity of organisms in producing H_2S is therefore of some importance in the reduction of selenite. This was studied in mannitol peptone medium and in depleted medium + cystine, since no H_2S was produced in depleted medium alone by any of the organisms tested. The results are shown in Table 4. It is noteworthy that the organisms which grew well in selenite broth were active H_2S producers in mannitol peptone medium and in depleted medium + cystine. *Escherichia coli* I did not produce H_2S in mannitol peptone medium but produced H_2S strongly from depleted medium + cystine. It would appear, then, that free cystine (or cysteine) was not present in mannitol peptone medium at the physiological stage of selenite reduction. Excess cystine must, therefore, exist as a component of complex substances (e.g. peptides) and the inability of *E. coli* I to produce H_2S in mannitol peptone medium may be due, perhaps, to the lack of enzymes necessary to free cystine in a suitable form for reduction.

Table 4. H_2S *production in mannitol peptone medium and depleted medium with cystine*

Thiamine was added to depleted medium for *Salmonella choleraesuis* strains. Complete blackening of surface of lead acetate paper represented by symbol + + + +; slight marginal blackening ±; intermediate degrees +, + +, and + + +; variable ( ). Incubated for 24 hr. at 37°.

	S. typhimurium	*E. coli* I	*C. freundii* I	*P. vulgaris*
Mannitol peptone medium	+ + +	–	+ + +	+ + + +
Depleted medium + cystine	+ + + +	+ + + +	+ + + +	+ + + +

	S. choleraesuis strains						
	Lab. strain	NCTC 5364	NCTC 5735	NCTC 6678	NCTC 5736	NCTC 5737	NCTC 5738
Mannitol peptone medium	–	–	–	+ + +	+ + +	+ + +	+ +
Depleted medium + cystine	–	(+)	(+)	(+)	+ +	(±)	(±)

When cystine was added to selenite broth there was a greater intensity of reduction of selenite with *Salmonella typhimurium* and with *Escherichia coli* I. Also, with *E. coli* I growth was greatly enhanced before selenite reduction took place. Strains of *S. choleraesuis* which required thiamine did not produce H_2S in mannitol peptone medium while the remaining strains were moderately active H_2S producers. All the strains of *S. choleraesuis* had little or no activity in depleted medium + cystine.

The relationship of sulphur metabolism to growth in selenite broth

The effect of adding different sulphur sources on the growth of organisms was studied. The more important findings have been collected in Table 5. It may be seen that in depleted medium + selenite, cystine was able to annul the toxic effect of the selenite on the growth of *Salmonella typhimurium* and *Escherichia coli* I, while methionine had no effect. Further information on the relationship of cystine and methionine to selenite was obtained with *Citrobacter freundii* I. No growth of *C. freundii* I was obtained in defined medium. The addition of methionine to the medium allowed a poor growth in 24 hr., but maximal growth was obtained when glutamic acid and glycine were also added.

No growth was obtained with cystine in 24 hr. In defined 'sulphur-free' medium the growth was poor when methionine was the sole sulphur source but maximal with cystine + methionine. Thus methionine with either sulphate or cystine would seem to be essential for optimal growth. With *C. freundii* I in defined medium + selenite growth occurred only with cystine + methionine. It is clear that even when methionine was required for optimal growth, cystine was necessary to overcome the toxic effect of selenite.

Table 5. *The effect of different sulphur sources on the growth of organisms in different media*

For *Citrobacter freundii* I defined and defined 'sulphur-free' medium were fortified with glutamic acid and glycine and for *Salmonella choleraesuis* with glutamic acid, aspartic acid, and thiamine. The strains of *S. choleraesuis* are not designated but the results are tabulated in the order shown in Table 3. Time of incubation 24 hr. at 37°. Inoculum 0·02 ml. × 10^{-3} of an overnight broth culture to 10 ml. of the medium. Growth is indicated by symbols as in Table 2.

		Sulphur supplements				
Organisms	Medium	Nil	Cystine	Methionine	Cystine + methionine	$K_2S_5O_6$
S. typhimurium	Depleted medium + 0·5 % (w/v) $NaHSeO_3$	−	+ + + +	−	.	.
E. coli I		−	+ + +	−	.	.
C. freundii I	Defined medium	−	−	+ + + +	+ + + +	.
	Defined 'sulphur-free' medium	−	−	+	+ + + +	.
	Defined medium + 0·1 % (w/v) $NaHSeO_3$	−	−	−	+ + + +	.
S. choleraesuis strains	Defined medium	+ +	+ + + +	+ + +	+ + + +	+ + +
		±	±	−	+	+
		+	+ + + +	+ + +	+ + + +	+ + +
		−	−	−	+	−
		+ +	+ +	+ + +	+ + + +	+ + +
		+ +	+ + +	+ + + +	+ + + +	+ +
		+ +	−	±	+	+ +
	Selenite broth	−	−	−	−	+ + + +
		−	−	−	−	−
		−	+ +	−	+ + + +	+ + +
		−	−	−	−	+ + +
		−	−	−	−	+ + +
		−	+ + +	−	+ + +	+ + +
		−	+ +	−	+ +	+ + +

The growth response of *Salmonella choleraesuis* strains to supplements of different sulphur sources was studied by using defined medium + glutamic acid + aspartic acid + thiamine. The quantity of pentathionate available was limited and was used almost entirely for studies with *S. choleraesuis*. Though the results (Table 5) were rather variable, nevertheless, cystine, methionine and pentathionate all made some contribution to enhancement of growth in defined medium. In selenite broth the most outstanding annulment of growth inhibition was obtained with pentathionate and not by cystine; methionine had no effect at all.

Growth studies with *Escherichia coli* I in selenite broth + different sulphur sources showed that pentathionate greatly enhanced growth, which was also improved by trithionate and dithionate, but to a much less extent. Investigation of growth of *Salmonella typhimurium* and *E. coli* I with very small inocula in defined medium and in defined 'sulphur-free' medium indicated that both organisms were able to utilize a wide range of sulphur sources and that growth was greatly enhanced by the addition of glutamic acid.

The role of seleno-polythionates in selectivity

Of the sulphur sources added to selenite broth sodium thiosulphate was of particular interest as it produced a strong precipitation of selenium and, provided its concentration was not too high, the medium allowed the growth of *Salmonella typhimurium* but completely suppressed the growth of *Escherichia coli* I. When equal volumes of 10 % (w/v) sodium thiosulphate and 10 % (w/v) sodium selenite were mixed there was a heavy precipitation of selenium which deposited overnight to leave a water-clear fluid. The solution, whenever the selenium deposit was removed by filtration, repeatedly precipitated selenium so that the presence of elementary selenium seemed to impart a stability to the solution. The addition of more thiosulphate to the solution had no effect, but when selenite was added there was an immediate precipitation of selenium. Selenite cannot, therefore, co-exist with thiosulphate; nevertheless, the addition of 0·5 ml. of the 10 % (w/v) solution to 10 ml. mannitol peptone medium produced a medium with practically the same range of selectivity as selenite broth. Mellor (1930) gave an account of the reaction between thiosulphate and selenium dioxide from the work of Norris & Fay (1900) and Heuer (1926). On the basis of these findings the reaction between sodium selenite and sodium thiosulphate is probably

$$(1)\quad NaHSeO_3+4Na_2S_2O_3+2H_2O = Na_2S_4O_6+Na_2SeS_4O_6+5NaOH$$

followed by

$$(2)\quad 2Na_2SeS_4O_6+6NaOH = 3Na_2S_2O_3+2Na_2SO_3+2Se+3H_2O.$$

From the chemical behaviour of the solution it would seem that rapid decomposition of sodium seleno-pentathionate occurs only in the absence of elementary selenium so that when a small amount of this compound has precipitated some selenium, reaction 2 is retarded.

The sodium tetrathionate found in reaction 1 probably decomposes rapidly and it is doubtful whether it contributes much, if anything, to the selective action of the mannitol peptone medium containing the thiosulphate + selenite solution. It is possible that other reactions given by Mellor (1930) may add to the complexity of the solution. For instance, tetrathionate may react with sulphite thus:

$$Na_2S_4O_6+Na_2SO_3 = Na_2S_3O_6+Na_2S_2O_3.$$

Trithionate may, however, combine with selenium to form sodium selenotetrathionate

$$Na_2S_3O_6+Se = Na_2SeS_3O_6.$$

Seleno-pentathionate may also combine with sulphite to form sodium seleno-trithionate which may then decompose as follows:

$$Na_2SeS_2O_6 = Se + SO_2 + Na_2SO_4,$$

or

$$Na_2SeS_2O_6 = Na_2S_2O_6 + Se.$$

It seems clear that the chemical interaction between sodium selenite and thiosulphate may result in an exceedingly complex solution of polythionates and seleno-polythionates as well as other sulphur compounds. Possibly seleno-polythionates act as inhibitory analogues of highly preferred sulphur compounds and of these, seleno-pentathionate may be the most important since pentathionate has been shown to annul the inhibition of growth of *Escherichia coli* I and *Salmonella choleraesuis* in selenite broth.

DISCUSSION

Selenite broth is not, as might be supposed, a simple peptone medium in which free selenite is present. The experimental evidence indicates rather that some of the selenite is bound, perhaps by the peptides, while the remainder enters into chemical reactions with other substances present in the medium. The importance of the peptone used in the preparation of the medium is therefore clear, and it would be reasonable to assume that the quality of the selenite broth might be adversely affected by a variety of factors such as a diminution of selenite-binding properties or differences in cystine content.

Reduction of selenite is a late manifestation of the growth of the culture and is due to the activity of organisms which had already grown before the selenite was reduced. Selectivity due to selenite appears to be associated with sulphur metabolism. Though different sulphur sources were found to enhance the growth of *Salmonella choleraesuis* and *Citrobacter freundii* I in defined medium, studies of *Escherichia coli* I and *S. typhimurium* did not reveal significant differences in sulphur metabolism. Roberts, Abelson, Cowie, Bolton & Britten (1955) found with *E. coli* that a large group of sulphur compounds allowed optimal growth, but by using an isotopic competition method they were able to show differences in preference for different sulphur compounds. When either sulphate or sulphite were used as the sole sulphur source the growth responses were identical, though in mixtures of the two the organism preferred sulphite to sulphate. Thiosulphate was more effective than sulphite in suppressing the utilization of sulphate; cystine was found to be the most highly preferred of all the sulphur sources examined. Methionine allowed a suboptimal growth and it was necessary to supply some other sulphur source in addition to obtain optimal growth. With *Torulopsis utilis* sulphite was a more effective competitor than thiosulphate, cystine was a poor competitor with sulphate and methionine almost completely suppressed the incorporation of sulphate sulphur. Cystine and polythionates can be included among the sulphur sources that may be highly preferred by given bacteria and the effect of adding cystine or pentathionate to selenite broth, with organisms usually suppressed or inhibited by this medium, is almost certainly due to the provision of a highly preferred sulphur source. This implies that on a nutritional basis cystine and pentathionate can annul the inhibitory effects of substances (e.g. selenite) which block sulphate utilization.

The work of Roberts *et al.* (1955) suggests an important difference in sulphur metabolism between bacteria in which cystine is the most highly preferred sulphur source to permit optimal growth and yeasts in which methionine is the most highly preferred sulphur source. Cystine annuls the toxicity of selenate for *Escherichia coli* (Fels & Cheldelin, 1949) and cysteine the toxicity of selenite (Opieńska-Blauth & Iwanowski, 1952). North & Bartram (1953) found that the addition of cystine to selenite broth enhanced the growth of *Salmonella typhimurium*. On the other hand, in yeast only methionine could annul the toxicity of selenate (Fels & Cheldelin, 1948). Of the organisms studied in the present investigation those able to grow well in selenite broth seemed to have a high preference for cystine and this might be a dominant factor in selectivity, though *E. coli* I may be able to utilize only free cystine. Methionine, on the other hand, though necessary for optimal growth in *Citrobacter freundii* I, does not seem to be important in sulphur competition or to play any part in selectivity due to selenite.

The importance of polythionates (in particular pentathionate) in the sulphur competition field has been stressed. Pentathionate is likely to be highly preferred since it is able to annul the inhibitory effect of selenite broth on *Escherichia coli* I and *Salmonella choleraesuis*. Cystine, however, can annul the inhibitory effect of selenite broth on *E. coli* I but has very little effect with *S. choleraesuis* in the same medium. From this it is concluded that while *E. coli* I may have a higher preference for cystine over pentathionate, *S. choleraesuis* has a higher preference for pentathionate over cystine. The significance of these findings lies in the capacity of selenite to react with sulphur compounds to produce seleno-polythionates which are presumably toxic compounds. Seleno-pentathionate, found as a result of chemical reaction between selenite and thiosulphate, may, for instance, act as an inhibitory analogue of pentathionate. Possibly other seleno-sulphur compounds may be found in selenite broth. Though the reactions that may occur in selenite broth are not known, this would seem to be a rational explanation for the selective action of selenite broth inasmuch as it provides an explanation not only as to why some species of bacteria can overcome the toxic effects of the medium but also why the growth of some species is suppressed.

I wish to express my thanks to Mr Gordon Weston of the Department of Chemistry and Dyeing, Bradford Institute of Technology, for advice on the seleno-polythionates and for kindly providing the polythionates used in this investigation.

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'Iron Transport' Compounds as Growth Stimulators for *Microbacterium* sp.

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SUMMARY: An organism, resembling *Microbacterium lacticum*, was found to be stimulated by liver or yeast extract in a medium composed of glucose, amino acids, vitamins, adenine, guanine, uracil, ammonium formate, and mineral salts. These complex materials could be replaced by comparable concentrations of porphyrin-containing compounds and by extremely low concentrations of ferrichrome, coprogen, or terregens factor, which are thought to act as intracellular 'iron transport factors'.

In 1952 the discoveries of three new organic compounds of biological interest were announced. These were coprogen (Hesseltine *et al.* 1952), ferrichrome (Neilands, 1952), and terregens factor (Lochhead, Burton & Thexton, 1952). Coprogen, an essential growth factor for the fungus Pilobolus, is produced by various bacteria (including actinomycetes) and fungi (Hesseltine *et al.* 1953). Ferrichrome was isolated from *Ustilago sphaerogena* and can replace coprogen in the nutrition of Pilobolus. Terregens factor is produced by *Arthrobacter pascens* and promotes the growth of *A. terregens*, a requirement which can also be met by coprogen and ferrichrome (Burton, Sowden & Lochhead, 1954). Neilands (1957), in his excellent review on microbial iron metabolism, has proposed a unified hypothesis to account for the biological activity of these three different chemical entities. According to Neilands, these growth factors may act as coenzymes for the intracellular transfer of iron in micro-organisms.

The importance of the 'iron transport' compounds in microbial metabolism would be considerably enhanced if their growth-promoting characteristics could be demonstrated in other microbes. Thus, we wish to describe experiments which show that these compounds are potent growth stimulators for an organism resembling *Microbacterium lacticum*.

METHODS

Identification of organism

The culture was isolated as a laboratory contaminant. For its identification, the following methods were used according to the Society of American Bacteriologists, Committee on Bacteriological Technic (1944): fermentations, indole production (Gnezda test), gelatin liquefaction, starch hydrolysis, and action on litmus milk. The procedures recommended by Smith, Gordon & Clark (1952) were used for the detection of gelatin hydrolysis, production of urease, production of nitrite from nitrate, production of acetylmethylcarbinol and casein hydrolysis. Catalase was detected by gas evolution upon addition of 3 % (v/v) H_2O_2 to a slant culture. Heat resistance was determined by harvesting cells grown in a 10 ml. liquid culture containing basal medium + 0·04 % injectable liver (Armour Laboratories, Kankakee, Illinois) for 2 days, adding 10 ml. sterile skim milk and dividing into several pre-heated tubes. The tubes were immersed in a water bath at 72°. At various times up to 60 min., tubes were withdrawn, cooled, and 0·1 ml. was spread on plates of basal medium containing liver + agar. The plates were incubated at 37° and examined after 3 days.

Acid production was determined by titration of liquid cultures with 0·01 N-NaOH using bromthymol blue. Lactic acid was estimated by the method of Barker & Summerson (1941).

Media

The basal medium used for the nutritional studies is shown in Table 1. The culture was preserved in lyophile tubes. Upon opening a tube, the cells were diluted in 0·02 M-K_2HPO_4 and spread on a large number of slants of basal medium containing 2·5 % (w/v) Bacto-agar and 0·04 % (w/v) injectable liver. These slants were stored at 5° and used when needed. When new slants were required they were prepared from another lyophile tube.

Table 1. *Composition of basal medium**

Component	Amount per litre (g.)	Component	Amount per litre (mg.)	Component	Amount per litre (μg.)
Glucose (autoclaved separately)	10	L-Histidine.HCl.H_2O	12	Biotin	10
		DL-Tryptophan	12	Folic acid	10
	(mg.)	Glycine	2·5	Lipoic acid	10
Ammonium formate	100	L-Cystine	2	Leucovorin	10
L-Glutamic acid	260	L-Hydroxyproline	1	B_{12}	2
L-Glutamine	120	Adenine.SO_4	10		(g.)
DL-Valine	79	Guanine.HCl	10	Na_2SO_4	2
DL-Aspartic acid	60	Uracil	10		(mg.)
DL-Leucine	48	Inositol	2	KCl	760
DL-Isoleucine	48	Calcium pantothenate	1	Na_2HPO_4	660
DL-Serine	48	Riboflavin	1	$MgCl_2.6H_2O$	420
L-Proline	44	Pyridoxine	1	NaCl	150
DL-Threonine	40	Pyridoxamine	1	$FeCl_3.6H_2O$	25
DL-Phenylalanine	39	Pyridoxal	1	$MnCl_2.4H_2O$	18
DL-Methionine	33	Nicotinic acid	1	$ZnCl_2$	10
L-Tyrosine	33	Thiamin	1		
L-Lysine.HCl.H_2O	32		(μg.)		
L-Asparagine	20	PABA	100		
DL-Alanine	19	Pantetheine	100		
L-Arginine.HCl	19				

* pH adjusted to 7·5 with NaOH.

For nutritional studies, the basal medium was prepared in double strength. Five ml. were added to each 20 by 175 mm. colorimeter tube followed by a calculated amount of distilled water. Small glass beakers (10 ml.) were used as closures for the tubes. The tubes were autoclaved at 120° for 15 min. and cooled to room temperature. One ml. of 10 % (w/v) autoclaved glucose was then added followed by the sterile additives. These were autoclaved except in the case of heat labile materials where sintered glass filters were employed. Inoculum was prepared by suspending the slant growth in 0·02 M-K_2HPO_4 and diluting to an optical density (O.D.) of 0·05, using the Lumetron colorimeter (model 402–E) fitted with a 660 mμ filter. One drop (0·05 ml.) of such a suspension was used to inoculate each tube. Incubation was carried out at 37° on a shaking machine imparting a rotary motion of 220 r.p.m. Duplicate tubes were used throughout the study.

Estimation of growth

Growth was measured after approximately 30 hr. with the Lumetron colorimeter. Uninoculated medium was used to set the instrument to an optical density of zero. When the O.D. was greater than 0·3, the tube contents were diluted with water and the O.D. was re-read. The O.D. figures given represent the product of the observed O.D. and the dilution factor.

RESULTS

Identification studies

Microscopic examination of growth from refrigerated slants showed clusters of Gram-positive 'cocci' similar to those of Micrococcus. A fresh slant was inoculated with such growth and examined at various ages. At 6 hr. the 'cocci' had developed into Gram-positive rods which were still present at 17 hr. By 40 hr. the culture was completely in the 'coccoid' stage again. Examination with the electron microscope revealed that the 'cocci' were really coccobacilli which elongated during early growth.

The organism was non-acid fast, non-sporeforming, and non-motile. Slant growth was yellow and glistening. The aerobic nature of the organisms was shown by its much more rapid growth under shaking conditions than in static culture.

Fermentation tests revealed that acid was produced from glucose, sucrose, maltose, galactose, fructose, mannose and mannitol. Lactose, xylose, rhamnose, sorbitol, glycerol and arabinose were not fermented but supported growth. No gas was produced.

The culture was catalase positive, but failed to produce urease, indole or acetylmethylcarbinol. Casein was not hydrolysed and there was no action on litmus milk. Nitrite was not produced from nitrate. Although gelatin was not liquefied, it was hydrolysed in the plate test. Starch was hydrolysed, but its fermentation was extremely weak. Under the conditions described above, the culture was not sterilized by 72° for 1 hr.

Acid production was quite weak under the static conditions used for the fermentation tests. In basal medium containing injectable liver, the culture produced 0·04 % acidity, calculated as lactic acid. About half of this was actually due to lactic acid according to the Barker–Summerson assay. On the other hand, under shaking conditions, there was much better growth and maximum acid production was 0·09–0·16 %. However, over 95 % of the acidity produced with aeration was due to acids other than lactic.

The data obtained indicate that the culture is a member of the genus *Microbacterium*. When this genus was examined by Doetsch & Pelczar (1948), they found that all the isolates were Gram-positive, non-sporeforming, non-acid fast, non-motile short rods which appear coccobacillary at times. Furthermore, all produced acid but not gas from glucose, fructose, and mannose but not from rhamnose and sorbitol, were catalase positive, and failed to liquefy gelatin, digest casein or form indole or acetylmethyl carbinol. In addition, our culture possesses high heat resistance and carries out a weak lactic acid fermentation, both characters usually being associated with Microbacterium.

The remainder of the fermentation results of the present study do not correlate exactly with any of the 18 isolates (representing three species) studied by Doetsch & Pelczar. However, these workers present data which show a considerable overlap of sugar fermentations among the species and quite a variation within species. Examination of the recent classification of the genus by Doetsch in the seventh edition of *Bergey's Manual* (1957) indicates the present culture resembles *Microbacterium lacticum* but differs from the standard description in its inability to ferment lactose.

Nutrition studies

When *Microbacterium* sp. was inoculated into the basal medium its growth was stimulated by addition of injectable liver and other liver preparations. Growth-promoting activity was not found in ribonucleic acid or deoxyribonucleic acid; however, yeast extract (Difco Laboratories Inc., Detroit, Michigan) and 'vitamin-free' enzyme-hydrolysed casein (Nutritional Biochemicals Corp., Cleveland, Ohio) were active. The possibility of a peptide requirement was explored by adding high levels (1–3 mg./ml.) of single amino acids to the basal medium (Peters, Prescott & Snell, 1953; Demain & Hendlin, 1958) but none of these supplements replaced liver.

A survey of approximately 200 compounds including purines, pyrimidines, nucleosides, nucleotides, organic acids, carbohydrates, intermediates of nitrogen and carbohydrate metabolism, sterols, amines and miscellaneous biochemicals revealed only one compound capable of replacing liver, i.e. cytochrome *c*. As shown in Fig. 1, cytochrome *c* was approximately as active as injectable liver. A further search for active materials was conducted with other porphyrin-containing compounds. The following compounds stimulated growth at levels below 50 μg./ml., but none was more active than cytochrome *c*: haemoglobin, haemin and protoporphyrin. Haematoporphyrin was inactive at 50 μg./ml. The porphyrin precursor, δ-amino laevulic acid, showed activity but again less than the cytochrome.

None of the above compounds was active enough to account for the liver response. The stimulation by haemin, however, provided a clue for further experimentation. It was recalled that in the nutrition of Pilobolus (Hesseltine *et al.* 1952) and *Arthrobacter terregens* (Burton *et al.* 1954) the relatively large haemin requirement could be replaced by low concentrations of coprogen and terregens factor, respectively. As shown in Fig. 2, coprogen was much more active than liver. Similar results were obtained with terregens factor and ferrichrome. The half-maximal figures for ferrichrome, coprogen and terregens factor concentrate were 1, 12 and 230 mμg./ml. respectively. In view of the observation by Burton *et al.* (1954) that the iron-chelating compound, aspergillic acid, was active for *Arthrobacter*, we tested this compound and found half-maximal activity at 60 mμg./ml. Furthermore, *N*-D-glucosylglycine ethyl ester was similarly active. This compound was tested when it was reported that fructose-phenylalanine was a potent chelator of the ferric

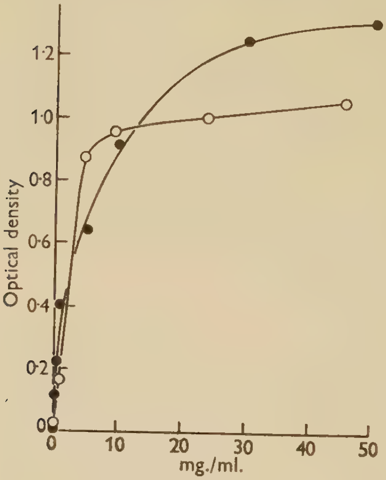

Fig. 1

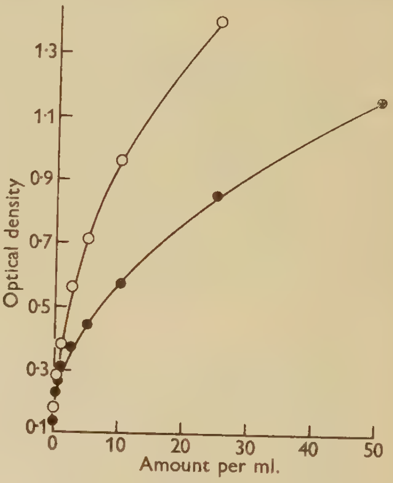

Fig. 2

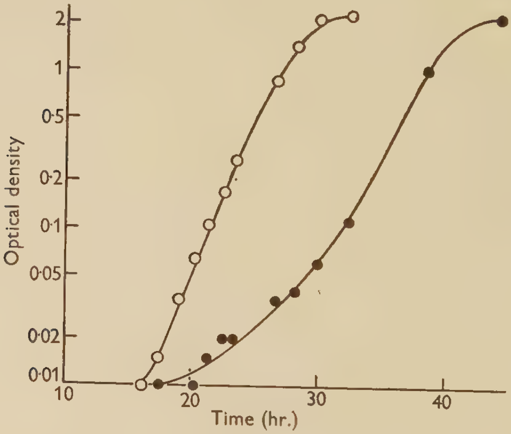

Fig. 3

Fig. 1. Activity of cytochrome *c* (●) and injectable liver (○) for growth of *Microbacterium* sp.

Fig. 2. Activity of millimicrogrammes of coprogen (●) and microgrammes of injectable liver (○) for growth of *Microbacterium* sp.

Fig. 3. Growth curves of *Microbacterium* sp. in the basal medium in the absence (●) and presence (○) of 100 mμg./ml. of ferrichrome.

ion (Neilands, 1957). Ethylenediamine tetraacetate and citrate showed no activity in our tests, indicating that the requirement is rather specific.

The nature of the growth stimulation was determined by comparing the growth curves of *Microbacterium* sp. in the presence and absence of ferrichrome. Figure 3 shows that the lag phase is shortened and that there is no marked effect on the rate or extent of growth.

DISCUSSION

The present study extends to the genus *Microbacterium* the nutritional requirement for 'iron transport factors'. Proof of the role of ferrichrome, coprogen, and terregens factor as coenzymes of iron metabolism, as suggested by Neilands (1957), must await refined biochemical studies; however, their importance increases as the list of organisms requiring such factors is enlarged.

The specificity of the requirement for our strain was shown by the lack of activity of citrate and ethylenediamine tetraacetate. Furthermore, the large number of compounds which were tested and found to be inactive, some of which were known chelating agents, speaks against a general requirement for chelating compounds. The specificity in the present work appears to be narrower, for example, than that observed for the autoclaved glucose-phosphate requirements in *Lactobacillus fermenti* (Ramsey & Lankford, 1956) and *Bacillus globigii* (Lankford, Kustoff & Sergeant, 1957). The inactivity of glucosyl-glycine with the latter organism also indicates that the glucose-phosphate effect is distinct from the 'iron transport factor' stimulation.

The activity of glucosyl-glycine in the present study suggests that 'iron transport factors' may also be required by *Lactobacillus gayoni*. Rogers, King & Cheldelin (1953) found that this compound could replace liver or yeast, which in turn replaced the stimulation obtained by autoclaving the components of the medium together. It is also of interest that glucosyl-glycine could replace the autoclaved glucose stimulation in *Propionibacterium freundenreichii* although high concentrations were required (Field & Lichstein, 1957). Similarly, Neilands (1957) has reported that autoclaved glucose-glutamate is an essential requirement for a strain of *Micrococcus lysodeikticus*. This requirement can be replaced by several compounds including fructose-phenylalanine and ferrichrome but not by ethylenediamine tetraacetic acid. Evidence is thus accumulating which indicates a connexion between compounds formed upon heating sugars with amino acids and 'iron transport factors'. The potentialities even become more interesting when one considers the data of Borsook, Abrams & Lowy (1955) concerning the role of ketose-amino acids in stimulation of amino acid incorporation into protein of rabbit reticulocytes. Fructose-L-glutamic acid, fructose-L-alanine and fructose-glycine were isolated from liver and could replace liver extract in the amino acid incorporation assay when low concentrations of iron were present. More recently, Kruh & Borsook (1956) demonstrated that fructose-amino acids were capable of stimulating haem synthesis and amino acid incorporation into globin.

The replacement of haemin by extremely low concentrations of 'iron transport factors' as observed with *Microbacterium* sp. was previously demonstrated with Pilobolus and *Arthrobacter terregens*. This repeatedly observed phenomenon led us to wonder whether the haemin requirements of other organisms, e.g. *Haemophilus influenzae* (Granick & Gilder, 1946) and isoniazid-resistant strains of *Mycobacterium tuberculosis* (Fisher, 1954) might also be met by these compounds. Recent experiments (Demain, unpublished) have eliminated the haemin-dependent *Escherichia coli* H7 (Beljanski, 1955) from consideration since coprogen, ferrichrome and terregens factor failed to replace haemin. Nevertheless, we feel that a requirement for haemin or cytochrome *c* should be investigated further before it is concluded that the primary defect is a deficient porphyrin-synthesizing system. It is quite probable that the porphyrin requirement is merely a result of a deficient intracellular ability to transfer or store low concentrations of iron.

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Nutritional and Related Biological Studies on the Free-Living Soil Amoeba, *Hartmannella rhysodes*

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SUMMARY: *Hartmannella rhysodes* Singh was cultivated under axenic conditions at 25–30° in a buffered physiological salt solution containing 1% (w/v) proteose peptone and a suitable additional carbon source. Optimal growth and maximum cyst formation were obtained aerobically under constant agitation in vessels coated with silicone. The addition of a thickening agent did not improve growth. No cysts were formed at low oxygen tensions. This may reflect a fundamental metabolic block in cyst formation. Growth curves were obtained with a variety of carbon sources: glucose, maltose and mannose supported optimal growth; lactose supported unusual growth which resembled diauxie. By testing for reducing sugars, no utilization of glucose was detected. A specific chemical test for glucose revealed that glucose was utilized during growth and by resting suspensions. Through the use of uniformly labelled ^{14}C glucose, it was possible to demonstrate that glucose was assimilated and also used as an energy source during growth. The osmotic concentration required for optimal growth was in itself not optimal when the total osmotic pressure was kept constant and the concentration of utilizable carbon sources was decreased.

Hartmannella rhysodes Singh is a small naked amoeba which forms non-reproductive cysts; it was isolated from soil and described by Singh (1952). It is placed in the family Hartmannellidae, class Sarcodina.

Physiological studies on small soil amoebae have yielded contradictory results. A requirement for an added carbon source, in addition to the basal medium, was described for a species of *Acanthamoeba* (Neff, 1957) and for *Mayorella palestinensis* (Reich, 1935). Evidence will be presented in this paper that this is also true for *Hartmannella rhysodes* and *A. castellanii*. However, neither Reich (1948, 1955) by respirometry nor Neff, Neff & Taylor (1958) by direct chemical analysis were able to show the use of glucose during growth. Cailleau (1933*a*, *b*, 1934) was unable to demonstrate either the need for, or utilization of, an added carbon source by *A. castellanii*. Operating within the restrictions imposed by the lack of a completely defined medium, the objectives of the present study were: (1) to study the utilization of different carbon sources, as measured by the growth response of *H. rhysodes*; (2) to examine by direct chemical analysis whether glucose was utilized as a carbon source; (3) to investigate some aspects of the general physiology of *H. rhysodes* as related to its life cycle.

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METHODS

Organism. Hartmannella rhysodes Singh and *Acanthamoeba castellanii* (Douglas) Volkonsky were obtained from Dr William Balamuth (Department of Zoology, University of California). The strains originated in the laboratories of Dr B. N. Singh and Dr A. Lwoff, respectively. *H. rhysodes* was the organism of primary interest; *A. castellanii* was used only in one experiment to demonstrate its growth response to glucose.

Culture procedures

Culture medium. The fluid culture medium used routinely and referred to as Amoeba Medium was modified from Neff's (1957) amoeba medium. The medium contained: 120 mg. NaCl; 3 mg. $MgCl_2.6H_2O$; 3 mg. $CaCl_2$; 3 mg. $FeSO_4$; 142 mg. Na_2HPO_4; 136 mg. KH_2PO_4; 10 g. Proteose Peptone (Difco); 18 g. glucose; water to 1 l.; final pH value 6·8.

In order to test the ability of different carbon sources to support the growth of *Hartmannella rhysodes*, the concentration of glucose in the Amoeba Medium was decreased to 0·0025 M. The carbon sources, as well as glucose control cultures, were then tested in this modification of the Amoeba Medium, at concentrations of 0·025, 0·05 and 0·1 M. Sucrose was added to the 0·025 and 0·05 M concentrations to increase their molar concentrations to 0·1 M.

The amoebae were tested in sodium acetate at pH values of 5·5–6·5. The phosphate buffer at the concentration used had no effect on the initial pH value of the medium. For this reason the pH value was adjusted with known amounts of 0·1 N-HCl. Since the total osmotic pressure was higher than that of unmodified Amoeba Medium and differed according to the desired pH value, they were brought to the same osmotic concentration with NaCl. Differences in acetate concentration were not brought to the same osmotic concentration. Parallel glucose cultures were tested in this modification of the Amoeba Medium at the pH values used.

Sterilization of sugars. All sugar solutions were sterilized by passage through a Millipore 'WH Microweb' membrane filter (Millipore Filter Corp.).

Manipulation of cultures. Both *Hartmannella rhysodes* and *Acanthamoeba castellanii* were isolated from their bacterial associates by migration on an agar surface. This technique was used by Oehler (1916, 1924) in isolating soil amoebae. Axenic fluid cultures were established by this procedure. Stock cultures were established in Amoeba Medium from a single organism, isolated by the migration technique from axenic cultures growing on solid surfaces. These cultures consisted of 50 ml. Amoeba Medium in 250 ml. Erlenmeyer flasks. They were subcultured monthly, with an inoculum of *c.* 0·5 % of the final yield of amoebae, and incubated statically at 30°. Mass cultures consisted of 200–400 ml. of Amoeba Medium in 1 l. Erlenmeyer flasks. These were incubated at 27° on a rotary shaker. The inocula varied from *c.* 10 to 25 % of the final yield of organisms. Growth experiments were carried-out with 10 ml. of Amoeba Medium in 50 ml. Erlenmeyer flasks. Inocula for growth experiments were *c.* 1 % of the final yield of organisms in a volume of Amoeba Medium not exceeding 0·5 % of the volume of the medium to be tested. All parallel experiments received the same inoculum in respect to size and source.

Aeration and agitation. Aeration and agitation were accomplished on a rotary shaker having a radius of $\frac{1}{4}$ in. at 115 rev./min. The growth vessels used under these conditions were coated with silicone (Dow Corning '200 Fluid', 350 c./s.) according to the manufacturer's instructions. All experiments were incubated at 27° on the rotary shaker unless otherwise stated.

Measurement of growth

Turbidity, measured by a 'Spectronic 20' colorimeter (Bausch & Lomb) at 600 mμ, was used to estimate the number of organisms unless otherwise stated. Optically matched test-tubes, attached at right angles to the side of the culture vessels above the level of the fluid, were used in growth experiments; percentage transmission was correlated with number of organisms both by counts and turbidity measurements performed over part of the growth cycle in parallel, and by dilution of a culture containing maximum log-growth organisms with fresh medium. To correct for concentration by evaporation, volumes were measured after growth, and the volume present at each measurement were calculated by assuming that the evaporation rate was linear. In order to minimize evaporation, the cotton-plugged culture vessels were capped with aluminium foil.

Analytical procedures

Sugar analysis. Two reducing-sugar tests were used for quantitative analysis of media: (1) the Nelson photometric adaptation of the Somogyi method (Nelson, 1944); (2) the anthrone method (Südhof, Kellner & Schulte, 1955*a*, *b*). A specific colorimetric test for glucose, using a preparation of glucose oxidase, supplied under the name 'Glucostat' (Worthington Biochemical Corp.), was also used.

Isotope and related procedures. Isotope experiments were carried out with uniformly labelled ^{14}C glucose furnished by Dr Michael Doudoroff (Department of Bacteriology, University of California). The original isotope was diluted with M-glucose solution, and added to sterile Amoeba Medium through a Swinny filter. Samples, dried on planchets under an infra-red lamp, were counted with a thin window counter and a decimal scaler for 10 min. intervals. Carbon dioxide was collected by isolating each experiment in an all-glass culture system, which was gassed periodically with CO_2-free air. The CO_2 was collected in CO_2-free 4 % (v/v) NaOH solution, which was made from a 40 % NaOH solution (sp.gr. 1·43) and CO_2-free water.

Dry weight; total carbon, hydrogen and nitrogen. Total nitrogen was determined by the Kjeldahl method (Steyermark, 1951). Total carbon and hydrogen determinations were performed by the combustion method (Steyermark, 1951). Dry weights were performed either by drying at 105–110° or in a desiccator over P_2O_5 at 30° with a slight vacuum.

RESULTS

Total nitrogen and dry weight

Amoebae harvested during the logarithmic phase of growth were used to determine the total nitrogen content as correlated to number of organisms. Total nitrogen determinations were performed on three separate cultures of amoebae, each in quadruplicate; glycine was used as a control. The results are presented in Table 1. The dry weight of organisms harvested during the logarithmic phase of growth was determined for washed organisms dried at 105–110°. Dry weight was correlated with number of organisms. The results (see Table 1) were determined from eight samples.

Table 1. *Total nitrogen and dry weight of* Hartmannella rhysodes *based on* 10^6 *organisms*

	Total nitrogen (mg.)	Dry weight (mg.)
Mean	0·0465	0·753
Range	0·0715–0·037	0·765–0·741
S.D.	0·0019	0·017

Measurement of growth by turbidity

The number of organisms was correlated with turbidity over part of the growth cycle. A satisfactory regression line was plotted by the method of least squares by correlating the log of number of organisms with the square root of % T; this correlation is presented in Fig. 1 and was found to be valid for samples derived by dilution. Periodical direct counts of organisms of all cultures measured by turbidity fell within the confidence interval for new observations.

Requirements for oxygen and surfaces

Oxygenation. In static deep cultures (2·5 cm.) no cysts were observed, although round forms resembling the precystic amoebae described by Ray & Hayes (1954) were observed. In shallow (1 cm.) cultures both cysts and round forms were seen. A comparison of maximum yields in deep and shallow cultures and in rotary agitated cultures is presented in Table 2. The data indicate that the growth of *Hartmannella rhysodes* was directly related to the degree of oxygenation and that cyst formation was inhibited at low oxygen tensions.

Surfaces. When the flasks used to grow amoebae under rotary agitation were not coated with silicone, poor or no growth was obtained. The silicone coating prevented the organisms from adhering to the surface of the vessel. The addition of sodium carboxymethyl cellulose at 0, 0·1, 0·5, 1·7 and 2·5 % (w/v) to stationary and agitated cultures did not improve growth. Thus the addition of a compound which forms micellar surfaces did not have any effect on growth; the amoebae do not appear to require a surface for growth and multiplication.

Anaerobiosis. Incubation in an atmosphere of hydrogen and *c.* 10 % (v/v)

CO_2, contained in a Brewer jar, was lethal. A few amoebae survived incubation under these conditions for short time periods (*c.* 24 hr.) although division and cyst formation were completely arrested.

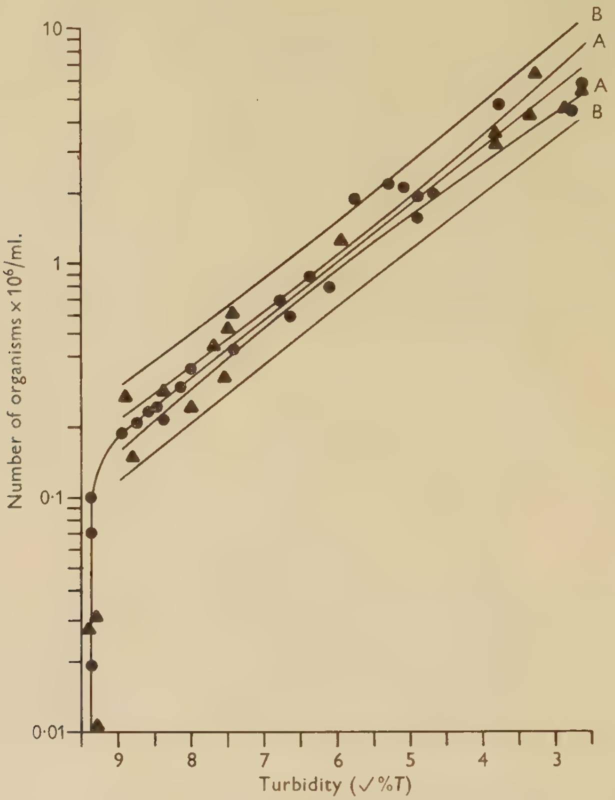

Fig. 1. Correlation of the number of organisms with turbidity. Confidence intervals are shown for the regression line (A) and for new observations (B) at the 95 % level. Each point represents a sample mean. Samples were derived from parts of the growth cycle (●) from which the regression line was plotted, and by dilution of a culture in maximum log growth (▲).

Table 2. *A comparison of the growth of* Hartmannella rhysodes *under different conditions of aeration*

	Maximum yield (× 10^6 organisms/ml.)	Time (days)
Stationary, deep (2·5 cm.)	1·13	40
	1·77	
	1·19	
Stationary, shallow (1 cm.)	1·88	26
	2·12	
	1·89	
Agitated by shaking (2·5 cm.)	3·3	10
	4·0	

Osmotic pressure

Osmotic pressure determination. The osmotic pressure of 1 % (w/v) Proteose peptone, determined by the method of Gortner & Harris (1914), is 0·05 osmolar; the calculated osmotic pressure for Amoeba Medium is 0·158 osmolar.

Osmotic pressure effect on growth. The effect of osmotic pressure on growth was determined by growing the amoebae in Amoeba Medium with different concentrations of glucose and sucrose in duplicate at 30°. Filter-sterilized sucrose did not support growth. The results (see Fig. 2) indicated that growth at all concentrations of glucose was depressed by increasing the osmotic pressure of the medium.

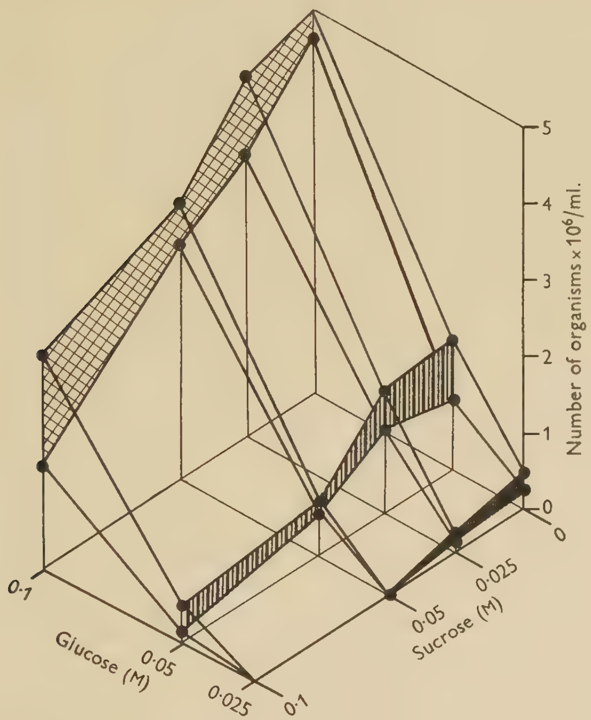

Fig. 2. Osmotic effect of sucrose on maximum yield for duplicate cultures.

Growth with different carbon sources

Known carbon sources. A comparison of the growth of *Hartmannella rhysodes* with various carbohydrates is shown in Table 3. Growth with sodium acetate and parallel glucose cultures is presented in Table 4.

Glucose, maltose or mannose supported the best growth. As seen in Fig. 3, growth with glucose was proportional to its concentration. The peculiar growth with lactose (Fig. 4) was not observed with any other carbon source; at all concentrations of lactose, the exponential growth phase was interrupted with a lag phase. No growth was observed with D-arabinose, D-raffinose,

Table 3. *Growth of* Hartmannella rhysodes *with various carbohydrates*

Organisms were grown at 27° on a shaker in Amoeba Medium containing the appropriate sugar in place of glucose. Data are presented as the mean of duplicate determinations. Mean generation time presented only where logarithmic growth is evident. Growth was measured turbidimetrically.

Carbon source	Concn. (M)	Maximum yield ($\times 10^6$ organisms/ml.)	Time required (days)	Mean generation time (hr.)
D-Glucose	0·1	5·8	14	42·1
	0·05	1·1	16	63·6
	0·025	0·1	18	—
D-Maltose	0·1	1·0	26	132
	0·05	3·7	28	57·6
	0·025	1·6	32	68·7
D-Mannose	0·1	2·4	13	51·8
	0·05	0·8	13	63·9
	0·025	0·3	19	155·2
D-Lactose	0·1	0·8	42	—
	0·05	1·5	46	—
	0·025	1	76	—
D-Ribose	0·1	0·2	36	132
	0·05	0·8	27	70
	0·025	0·5	36	86·7
D-Fructose	0·1	—	—	—
	0·05	0·9	18	80·4
	0·025	0·3	22	101·4
D-Xylose	0·1	—	—	—
	0·05	0·7	34	—
	0·025	0·5	41	166·5
D-Glucosamine HCl	0·1	—	—	—
	0·05	—	—	—
	0·025	0·6	48	—
D-Galactose	0·1	—	—	—
	0·05	0·2	27	151
	0·025	0·4	32	108·5
D-Lyxose	0·1	—	—	—
	0·05	—	—	—
	0·025	0·2	38	—
L-Rhamnose	0·1	—	—	—
	0·05	0·2	39	—
	0·025	0·1	45	—

i-erythritol, D-sorbitol or dulcitol. Optimum growth with sodium acetate occurred at pH 6·5 and 0·025 and 0·05 M, which coincides with the optimum pH value for growth with glucose.

Effect of Proteose peptone and yeast extract on growth. Concentrations of Proteose peptone greater than 1 % (w/v) supported the growth of *Hartmannella rhysodes* in the absence of an added carbon source. At 2 % (w/v) Proteose peptone, the maximum yield was less than $0·1 \times 10^6$ organisms/ml. No growth was detected by turbidimetry with 1 % (w/v) Proteose peptone. Yeast extract was also capable of supporting very poor growth in the absence of an added carbon source. A maximum yield of less than $0·1 \times 10^6$ organisms/ml. was obtained with 0·1 % (w/v) yeast extract.

In Amoeba Medium containing 0·1 M-glucose, Proteose peptone and yeast extract affect the maximum yield of amoebae (Fig. 5), but did not alter the generation time. In the presence of 0·025 M-glucose, the generation time (and maximum yield) increased with increasing concentrations of yeast extract and Proteose peptone (Fig. 5).

Table 4. *Growth of* Hartmannella rhysodes *with sodium acetate*

Organisms were grown at 27° on a shaker in Amoeba Medium containing sodium acetate as the carbon source. The pH was adjusted with known amounts of HCl. Data are presented as the mean of duplicate cultures. Growth was measured turbidimetrically.

	pH (initial)	Maximum yield ($\times 10^6$ organisms/ml.)	Time required (days)
0·1 M-NaAc	5·9	—	—
	6·2	—	—
	6·4	0·4	24·5
	6·6	0·5	31
0·05 M-NaAc	5·8	0·4	31
	6·1	0·4	28·5
	6·3	0·3	26
	6·6	0·4	27
0·025 M-NaAc	5·7	0·4	27
	6·0	0·3	28
	6·2	0·3	23·5
	6·5	0·5	23
0·05 M-Glucose	5·4	2·3	32·5
	5·9	1·9	28
	6·1	1·9	23
	6·5	2·3	23

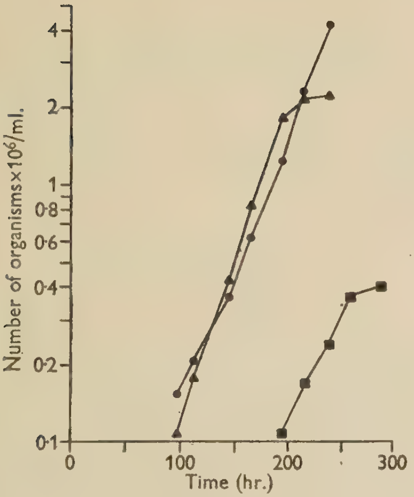

Fig. 3

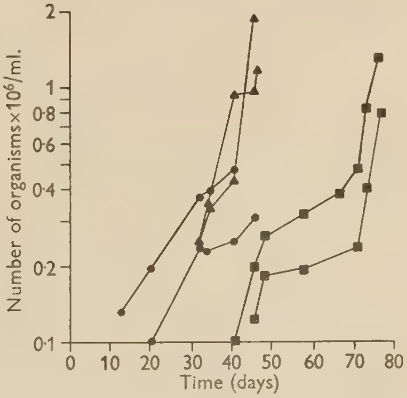

Fig. 4

Fig. 3. Growth with glucose at 30° on a shaker. Each point represents the mean of duplicate cultures with glucose concentrations of 0·1 M (●), 0·05 M (▲) and 0·025 M (■).

Fig. 4. Growth with lactose at 27° on a shaker. ●, 0·1 M; ▲, 0·05 M; ■, 0·025 M.

Growth response of Acanthamoeba castellanii *to glucose.* The growth response of *A. castellanii* to different glucose concentrations in Amoeba Medium was tested at 30° in stationary cultures. The results (Table 5) indicate that *A. castellanii* responded to different glucose concentrations in a manner similar to *Hartmannella rhysodes*. The organism differed from *H. rhysodes* in exhibiting slight growth (approximately $0{\cdot}1 \times 10^6$ organisms/ml.) in the presence of 1% (w/v) Proteose peptone without an added carbon source.

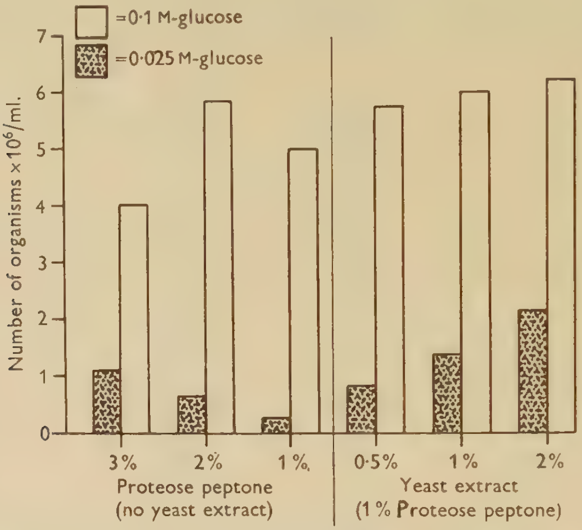

Fig. 5. Effect of Proteose peptone and yeast extract on growth in the presence of glucose. Shown as the average maximum yields of duplicate cultures.

Table 5. *Growth of* Acanthamoeba castellanii *with glucose*

Organisms were grown at 30° in static cultures of Amoeba Medium. Growth was measured turbidimetrically.

Glucose concn. (M)	Maximum yield ($\times 10^6$ organisms/ml.)	Time required (days)
0·1	0·91	27
	0·52	24
0·05	0·43	12
	0·43	18
0·025	Less than 0·1	12
	0·155	16

Utilization of glucose

Direct demonstration of glucose utilization after growth. Analysis of *Hartmannella rhysodes* cultures by the Nelson modification of the Somogyi test for reducing sugars did not reveal any significant change in reducing sugar concentration after growth in Amoeba Medium. The results of an analysis of duplicate cultures after exponential growth, by the glucose oxidase test and the anthone test for reducing sugars, are given in Table 6 as the mean of duplicate determinations and in Fig. 6 as percentage of the glucose initially present. The results indicate that glucose was used during growth, but a reducing substance was produced which reacted with the anthrone.

Direct demonstration of glucose utilization by resting organisms. The organisms (45 to 50×10^6/ml.) were placed in 50 ml. silicone-coated Erlenmeyer flasks with 5 ml. test solution. The test solutions were composed of 5·8 g. NaCl; 3 mg. $MgCl.6H_2O$; 3 mg. $CaCl_2$; 3 mg. $FeSO_4$; 142 mg. Na_2HPO_4; 136 mg. KH_2PO_4; water to 1 l.; this will be designated as Amoeba Saline. To the Amoeba Saline were added different concentrations of glucose. The results of uptake of glucose by resting organisms as determined by the glucose oxidase test are plotted in Fig. 7. All experiments were done in quadruplicate for

Table 6. *Sugar analysis with glucose oxidase and anthrone of* Hartmannella rhysodes *cultures after exponential growth*

Specific determination of glucose was accomplished with glucose oxidase ('Glucostat'). Anthrone was used as a test for reducing sugars.

Original concn. (mg./ml.)	Amount of glucose used (mg./ml.)		Incubation time (days)	Maximum yield ($\times 10^6$ organisms/ml.)
	Glucose oxidase	Anthrone		
27	3·3	3·36 incr.	10	5·4
18	4·5	0·2 incr.	11	2·1
9	4·04	0·7	11	2·1
4·5	1·58	1·1	13	0·6

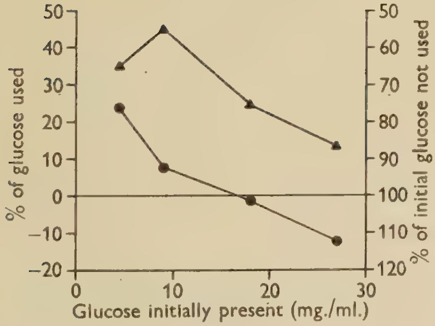

Fig. 6

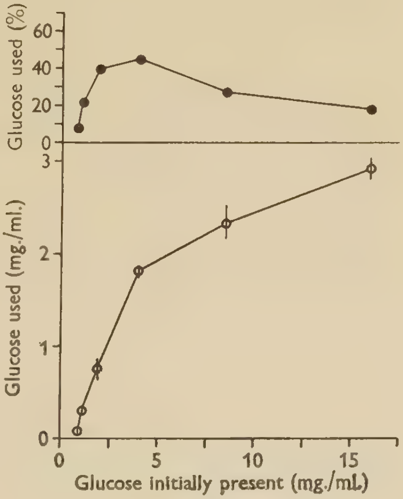

Fig. 7

Fig. 6. Percentage of initial concentration of glucose used after exponential growth, determined by glucose oxidase (▲) and anthrone (●).

Fig. 7. Uptake of glucose by 50×10^6 resting organisms/ml. after a 24 hr. period. At each concentration four suspensions were tested with glucose oxidase; the results given as the mean and range of these determinations.

a 24 hr. period. Anthrone determinations after 24 hr. indicated an approximate doubling of the reducing sugar concentrations. The medium became alkaline (pH 8·0–8·2) in all cases. Counts before and after experiments indicated that no appreciable cytolysis of organisms took place. Cysts were not observed after incubation for 24 hr.

Distribution of uniformly labelled glucose ^{14}C. Amoebae were grown for 5 days in 10 ml. Amoeba Medium with labelled 0·1 M-glucose having a specific activity of 442·5 counts/min./mg. carbon. During growth all of the CO_2 was collected. After growth the medium was acidified (pH 5) with KH_2PO_4, and the residual CO_2 was collected. The amoebae were then harvested, washed in Amoeba Saline, suspended in 1 ml. Amoeba Saline and returned to the 50 ml. incubation flask for 24 hr. All of the CO_2 was collected from the resting organisms. Dry weights of organisms were determined before and after starvation. Glucose concentrations of culture medium before and after growth, and of Amoeba Saline, were determined by the glucose oxidase test.

Of the glucose used, 24·1 % was assimilated, 40 % was returned to the medium as compounds other than glucose and 52·8 % was converted to CO_2. The error in ^{14}C recovery (5 %) and the error in the glucose determinations (11·9 %), before and after growth, account for a total in excess of 100 %. Assimilated carbon from glucose contributed 21·1 % of the total carbon in the amoebae. Glucose carbon contributed to 76·4 % of the total CO_2 produced. After starvation for 24 hr., the amoebae lost 57·7 % of their dry weight, which included 6·95 % of carbon derived from glucose lost to the medium and 29·4 % lost to the CO_2. Of the glucose carbon returned to the Amoeba Saline, 54 % was glucose. The specific activity of the amoeba carbon increased from 93·5 to 111 counts/min./mg. carbon.

Weights of cell fractions. In order to characterize partially the chemical components of the organism which contributed to the endogenous respiration, organisms were fractionated and weighed. Fresh and starved organisms were washed in saline and distilled water in weighed centrifuge tubes, dried over P_2O_5 and weighed. The organisms were then extracted twice for 20 min. with 2 vol. of cold 5 % (w/v) trichloroacetic acid (TCA), followed by a wash with 1 vol. of cold 1 % (w/v) TCA. The precipitate was dried over P_2O_5 and weighed. It was then extracted with 50 vol. of ethanol + ether (3:1, v/v) in three batches, dried over P_2O_5 and weighed. The results, presented in Table 7, indicate that the TCA-soluble fraction was responsible for most of the loss of dry weight from starved cells.

Carbon, hydrogen, oxygen and nitrogen determinations. The carbon, hydrogen, oxygen and nitrogen contents of fresh and starved organisms were determined by the direct analysis of all components except oxygen, whose content was calculated after correction for ash weight. The results are presented in Table 8.

Production of reducing compounds. A reducing substance which reacts with anthrone has already been described above for cultures containing glucose, as well as for resting organisms in the presence of glucose. Reducing compounds were also produced by cultures in which sodium acetate or glycerol served as added carbon sources; other added carbon sources were not investigated.

Organisms were harvested, washed and starved in Amoeba Saline under shaking conditions for 72 hr. Every 24 hr. they were transferred to fresh saline. After 24 hr. cytolysis began to become noticeable, so that at the end of the 72 hr. period, one-half of the original number was present (20 to 25×10^6 organisms/ml.). The Molisch reaction of equal intensity was obtained with samples of cell-free saline taken after all three time periods. There was no reaction with anthrone after the first 24 hr. period.

After 24 hr. of starvation, 45 to 50×10^6 organisms/ml. were incubated with 0·1 M-glucose with 20, 10, 5 and 0 mg./ml. Proteose Peptone. Analysis by glucose oxidase and anthrone failed to reveal any effect of Proteose Peptone on glucose uptake or on the production of reducing compounds.

Table 7. *Dry weights of cell fractions*

Precipitate dried over P_2O_5. Soluble fraction calculated from the loss of dry weight. Cells starved for 24 hr.

	Fresh organisms, % total weight	Starved organisms, % total weight
TCA soluble	53	44
TCA precipitate	47	56
Alcohol + ether soluble	24	23·8
Extracted precipitate	22·6	32

Table 8. *Carbon, hydrogen, oxygen and nitrogen content of* Hartmannella rhysodes

Total nitrogen determined by Kjeldahl method; total carbon-hydrogen determined by combustion; total oxygen calculated by difference after correction for ash weight.

	% C	% H	% O	% N
Fresh organisms	46·9	7·41	30·3	10
Starved organisms	47·2	7·15	29·2	10·25

DISCUSSION

Carbon sources

Utilization of known carbon sources. The only investigators who have used quantitative methods for describing the suitability of various carbon sources to support the growth of soil amoebae are Neff *et al.* (1958). Their data are presented in terms of yield, without regard to generation time, from cultures harvested after 2 weeks of incubation. Under these conditions there is no criterion by which to assume that the cultures were harvested at maximum growth. Neff *et al.* (1958) autoclaved all of the sugars in the presence of medium at an acid pH value. Although all the products of this operation are not predictable, there is no doubt that sugars are not stable (Davis & Rogers, 1939). Both *Hartmannella rhysodes* (from the results presented here) and Neff's *Acanthamoeba* sp. (Neff *et al.* 1958) use glucose, maltose, mannose, lactose, ribose, fructose, and galactose, neither uses arabinose, dulcitol and sorbitol. Only *H. rhysodes* uses xylose and rhamnose. Only *Acanthamoeba* sp.

(Neff *et al.* 1958) uses sucrose and raffinose. It would be interesting to see whether unautoclaved sucrose supports the growth of *Acanthamoeba* sp. since *H. rhysodes* can grow with sucrose as carbon source when the medium containing sucrose is autoclaved. Glucose, maltose and mannose support the best growth of *H. rhysodes*.

Neff *et al.* (1958) found optimal growth in 0·05 M-sodium acetate at pH 6, whereas in glucose the optimum was pH 5·6. Cultures of a 'given series' were harvested at the same time. If this means all acetate cultures were harvested at one time, and that all glucose cultures were harvested at one time, then there is no criterion for assuming that their results are based on maximum yields. *Hartmannella rhysodes* grows best with 0·025 and 0·05 M-sodium acetate at pH 6·5, which coincides with the optimum pH value for growth with glucose.

The irregular growth obtained in lactose (Fig. 4) is similar to a diauxic growth curve. If this is true, then lactose is probably broken down to glucose and galactose, one is used and after a lag phase the other is used.

Role of Proteose peptone and yeast extract. The results presented here demonstrate that Proteose peptone and yeast extract served as carbon sources for *Hartmannella rhysodes* (Fig. 5), and Proteose peptone (yeast extract not tested) in the case of *Acanthamoeba castellanii* (Table 5). The experiment with uniformly labelled ^{14}C glucose indicates that Proteose peptone carbon contributed 24 % of the CO_2 produced by the amoebae and 79 % of the assimilated carbon. These results may be used as a possible explanation of Cailleau's (1933*a*, *b*) finding that *Acanthamoeba castellanii* grew in a minimum of 3 % peptone and that added sugars had no effect on growth. Although no data are available, the carbon content of the 3 % peptone used by Cailleau was undoubtedly higher than that of 1 % Proteose peptone. Since Cailleau's cultures were limited by the concentration of peptone, the limiting factor or factors may have served some function other than that of a carbon source. Under these conditions, added carbon sources might not have any noticeable effect on growth.

The shift to an alkaline pH value and the failure of other workers (Cailleau, 1933*b*; Neff *et al.* 1958) to demonstrate the disappearance of sugars from media after growth has led to the speculation by others that nitrogenous compounds serve as a primary carbon source of these small soil amoebae. However, in view of the observations presented here, as well as the fact that peptone digests contain more than just nitrogen compounds, it does not seem likely that nitrogenous compounds are used preferentially as a carbon source.

Utilization of glucose. There has been no direct evidence of carbohydrate utilization in small soil amoebae. Reich (1948) was unable to demonstrate any effect of glucose on the respiration of *Mayorella palestinensis* in washed suspensions; Neff *et al.* (1958) have also found this with *Acanthamoeba* sp. In both cases, however, glucose or some other added carbon source in addition to Proteose Peptone was required for growth. From the fractionation studies presented here on fresh and starved organisms (Table 7), the TCA-soluble fraction appears to be the main site of endogenous respiration. The increase in specific activity of the total carbon of the organism after endogenous respiration indicates that more of the organism's components derived from carbon sources other than glucose are lost than those derived from glucose.

Cailleau (1933*b*) was unable to demonstrate any change in reducing sugar content when glucose was added to cultures of *Acanthamoeba castellanii*. Neff *et al.* (1958) were also unable to demonstrate a decrease in reducing sugar content of medium, in spite of the fact that *Acanthamoeba* sp. requires it for growth in the presence of 1 % Proteose Peptone. Neff *et al.* (1958) were able to demonstrate a decrease in the reducing content of culture medium after 35 weeks of incubation. They were also able to demonstrate after 3 days of incubation a 35 % decrease in reducing value of a washed suspension containing approximately twice the maximum yield of organisms and 0·05 M-glucose. From their respirometry and resting-cell data, it can be calculated that less than 1 % of the CO_2 would come from the added carbon source, if all of it were used for respiration.

The data presented here on the changes in reducing-sugar concentration and glucose concentration of culture media (Table 6, Fig. 6) indicate that Neff *et al.* (1958), by determining glucose concentrations with a reducing sugar test, did not measure the disappearance of glucose but rather the total reducing-sugar content of the medium. It is possible to demonstrate (Table 6, Fig. 6) that culture media containing suboptimal amounts of glucose show a moderate decrease in reducing sugars after growth. The specific analysis for glucose in parallel with reducing-sugar determinations (Table 6, Fig. 6) demonstrates that glucose is used in appreciable quantity. The labelled glucose experiment demonstrates that glucose is utilized as a carbon source for energy and assimilation. It is also of interest to note from this experiment that a large portion of the glucose is returned to the medium in an altered form.

Diffusion barrier. The requirement for large amounts of glucose in proportion to the amount used by growing organisms suggests a diffusion problem. The amount of glucose used by washed organisms (Fig. 7) is proportional to the concentration in the range in which the mean generation time of growing organisms is similar. This suggests that if glucose is transported actively into the cell, the carrier is not yet saturated and that passage is still dependent on the external concentration of glucose. An alternative would be that glucose uptake is accomplished by diffusion.

The possibility of a diffusion problem is also supported by the observation that there is no optimal osmotic concentration at the concentrations tested (Fig. 2). Since the concentrations tested fell within the range of optimal growth, it may be assumed that osmotic concentrations necessary for optimal growth are above the real optimal osmotic concentration. If this be true, then the amoeba has adapted itself to living in environments of high osmotic concentration by forming a diffusion barrier. In its natural environment this barrier would not present any difficulties since it would be feeding on particulate matter such as bacteria. Furthermore, although the amoebae are found in soil and environments of high osmotic pressure such as faeces and sewage, it is possible that a satisfactory natural environment might be fresh water.

The mean generation time for *Hartmannella rhysodes* under optimal conditions was 26 hr. This is similar to the minimum mean generation time for *Acanthamoeba* sp. (Neff *et al.* 1958). In comparison to other protozoa, the mean generation times for these amoebae are exceptionally long. This may mean that the experimental conditions for the cultivation of these amoebae were not optimal, or that this is one of the inherent properties of the small soil amoebae. Although no quantitative data are available the mean generation times of these amoebae appear to be much shorter in the presence of bacteria (and a lower osmotic pressure). It would thus appear that the media used for the cultivation of these amoebae under axenic conditions was not optimal.

Oxygen requirement and the influence of surfaces

Aeration. The results indicate that *Hartmannella rhysodes* is an obligate aerobe. Attempts by others to aerate cultures of hartmannellids have yielded confusing results. According to Storm, Hutner & Cowperthwaite (1951), *H. rhysodes* and *Acanthamoeba castellanii* are indifferent to aeration. Neff *et al.* (1958) found that aeration by swirling suppressed growth as compared to equivalent static cultures of *Acanthamoeba* sp. However, Neff *et al.* (1958) found that shallow cultures supported better growth than deep cultures and that aeration by bubbling supported the best growth.

If organisms which adhere to the glass surface of culture vessels are ruptured when the vessel is swirled, then the results quoted above can be explained on the basis of surface sensitivity. Neff *et al.* (1958) used silicone grease to prevent the organisms from sticking to glass surfaces in vessels used for direct counts, but did not coat growth vessels. The method of aeration used by Storm, Hutner & Cowperthwaite (1951) was not mentioned.

Cysts have never been seen in deep standing cultures, whereas in equally deep agitated cultures or in shallow standing cultures, cysts were formed. In all cases round forms were seen, identical with those described by Ray & Hayes (1954) as precystic amoebae. Since the only variable is the degree of aeration, it would seem that a final step in encystation requires optimal aeration.

Effect of agents to increase viscosity. Balamuth (1957) reports that silica gel or agar improved the growth of *Entamoeba invadens*, and that this action was independent of the reducing condition of the medium. No evidence was found by me, that addition of sodium carboxymethyl cellulose to increase the viscosity was beneficial to the growth of *Hartmannella rhysodes*. If it be assumed that the results reported here indicate that amoebae do not require a surface for growth, then any benefit derived from the addition of an agent to increase viscosity might be due to the creation of more surfaces for the amoebae to distribute themselves in the absence of agitation.

This study is part of a dissertation submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Zoology at the University of California, and was conducted under the direction of Professor William Balamuth. Grateful acknowledgement is hereby extended also to Professor Michael Doudoroff and to Professor Morgan Harris for advice and material support given during the course of the investigation. The last year of study was greatly aided by award of a U.S. Public Health Service Predoctoral Fellowship of the National Institutes of Health.

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Alkali Resistance in a Strain of *Bacillus cereus* Pathogenic for the Larch Sawfly *Pristiphora erichsonii*

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SUMMARY: Upon repeated subculture in media of gradually increasing pH value a strain of *Bacillus cereus* developed the ability to grow under strongly akaline conditions. Organisms with the highest alkali resistance could grow at pH 10·3, and stocks of intermediate resistance were also obtained. When growing on alkaline agar, resistant bacteria underwent marked morphological changes. In liquid media, the bacteria retained the ability to synthesize lecithinase. Resistance was not lost upon several transfers on a neutral medium.

Resistant bacteria when growing in alkaline media caused a rapid decrease in pH value. A similar pH decrease was caused by sensitive bacteria in media of the highest degree of alkalinity in which they could grow. The decline of pH value accompanied, but did not precede, growth; acid production by the resistant bacteria does not appear to be a primary mechanism of resistance.

Most bacterial species grow best in media of a relatively restricted pH range, near neutrality. *Topley and Wilson's Principles* (1955) stated that, with few exceptions, human pathogens grow in the range pH 5·0–8·0. To the extent that the pH range for growth of other bacterial species has been studied, there appear to be few major deviations from these figures. The most striking, on the acid side, is that of *Thiobacillus thiooxidans*, which can grow in '5–7 %' H_2SO_4 (Umbreit, 1951). On the alkaline side, *Streptococcus faecalis* was reported to grow up to pH 11, as well as in strongly acid media (Downie & Cruickshank, 1928). The effect of the pH value of the medium on the growth of *Bacillus cereus* Fr. & Fr. is one of the factors governing the pathogenicity of this organism for insects. In recent discussions of this subject (Heimpel, 1955*a*; Heimpel & Angus, 1958) it was pointed out that *B. cereus* ingested by mouth is a pathogen of larvae of the codling moth, *Carpocapsa pomonella* L. (Stephens, 1952), and of the larch sawfly, *Pristiphora erichsoni* Htg. (Heimpel, 1954), but not of the forest tent caterpillar, *Malacosoma disstria* Hbn. In the first two insects the midgut pH value is near neutrality and within the growth range of normal *B. cereus*. In the last organism, however, the midgut is at pH 9·5–10·5, a range outside that in which *B. cereus* usually grows.

The work described here arose from an attempt to increase the host range of micro-organisms which infect insects. For *Bacillus cereus* to cause disease in insect larvae the following sequence of events appears necessary: the bacteria or their spore must be swallowed; the spores, when present, must germinate; and the vegetative organisms must grow and damage the insect, possibly by lecithinase production (Heimpel, 1955*b*; Kushner & Heimpel, 1957). By whatever mechanism the bacteria kill the insect, in most cases it is likely that they must first multiply inside it. Surveys of internal pH values of insects (Staudenmayer, 1940; Waterhouse, 1949) show that the larvae of several species of phytophagous lepidopterous insects have, as does the forest tent caterpillar, highly alkaline midgut contents. Alkaline midgut conditions have also been observed in the Trichoptera and in some species of Coleoptera. Although *B. cereus*, as well as many other bacteria, would not normally be expected to grow in these insects, it seemed possible that bacteria induced to grow under more alkaline conditions than usual might be able to grow in these insects and perhaps to kill them. As a first step in this work, we have produced an alkali-resistant strain of *B. cereus*, which is able to grow in media of higher pH value than can the parent strain.

METHODS

Organisms. Bacillus cereus, strain Mu-3055, a pathogen of the larch sawfly (Heimpel, 1955*b*), was used throughout this study.

Growth media. Buffered growth media were used in all experiments, the buffer concentrations chosen being the highest that would permit maximal bacterial growth. The following media were prepared:

(1) Nutrient broth + phosphate buffer. Phosphate buffer (0·1 M, pH 7·5) was prepared by dissolving 71·6 g. $Na_2HPO_4 . 12H_2O$ in water, adding 3·2 ml. conc. HCl, and bringing the volume to 2 l. with water. Dried nutrient broth (Difco) was dissolved in this to 8 g./l. The final pH value was 7·4.

(2) Nutrient broth + tris buffer. A 0·05 M solution of 2-amino-2-hydroxymethylpropane-1:3 diol (tris: pH 7·5) was prepared by dissolving 12·1 g. tris in water, adding 6·25 ml. conc. HCl, and diluting to 2 l. Nutrient broth (dried, Difco) was made up in this, as for medium no. 1, the final pH value being 7·5.

(3) Casein hydrolysate + tris buffer. A 2 % (w/v) solution of 'Enzymatic Casein Hydrolysate' (Nutritional Biochemicals Co., Cleveland, Ohio, U.S.A.) was made in tris buffer solution as in medium no. 2. The final pH value was 7·5.

(4) Buffered nutrient agar. Nutrient agar (Difco) was dissolved in 0·1 M-phosphate buffer; or 1·5 % (w/v) 'Bacto Agar' (Difco) was made up in nutrient broth + phosphate buffer.

For most of this work the liquid media were dispensed in 10 ml. lots in 1 in. × 8 in. culture tubes and autoclaved at 15 lb./sq.in. for 15 min. Immediately before use, the pH value of a tube of medium was brought to the desired level by adding N- or 10 N-NaOH, as given by previously determined titration curves. The buffered agar was sterilized in 5 or 10 ml. lots in screw-cap tubes. For use, the agar was re-melted by immersing the tubes in a hot water bath, and NaOH was added before making slopes or pouring plates. Addition of NaOH *after* sterilizing was necessary since autoclaving strongly alkaline media led to charring. It was necessary, furthermore, to adjust the pH value of each tube just before use, since these media do not maintain a high pH value indefinitely. The extent of the decrease in pH value observed when different samples of sterile alkaline media were shaken at 37° is shown in Table 1. Alkaline casein hydrolysate + tris buffer medium was the most stable and alkaline nutrient broth + tris buffer medium the least so. Media of pH 8·0 or less did not change pH value on incubation in the absence of bacteria. The chemical changes which caused the pH to decrease were not investigated, but CO_2 absorption was probably involved.

Table 1. *Fall of* pH *in sterile alkaline media incubated at* 37°

Time (hr.)	pH									
	Nutrient broth + phosphate			Nutrient broth + tris				Casein hydrolysate + tris		
0	9·36	9·84	10·39	9·48	10·00	10·51	11·05	9·61	10·00	10·47
10	9·22	9·72	10·38	9·36	9·77	10·18	10·70	9·50	9·99	10·34
20	9·05	9·47	10·01	9·28	9·51	9·74	10·08	9·48	9·98	10·23
30	9·03	9·37	10·01	9·23	9·52	9·72	10·02	9·42	9·85	10·15

All media of initial pH 7·40 did not change pH on incubation.

In following bacterial growth the culture tubes were shaken on a rotary shaker in an incubator at 37°. The rate of shaking was 160 cycles/min. and each tube moved in a circle of diameter 2 in. Growth was measured directly in the culture tubes as turbidity, the changes in optical density at 650 $m\mu$ being followed by a Beckman Model DU spectrophotometer fitted with a test-tube adapter. In this system an optical density of 1·0 corresponded to 0·32 mg. dry wt. bacteria/ml. or to 8×10^7 viable bacteria /ml. as measured by plate counts. All figures given are corrected for the turbidity caused by the inoculum itself.

Measurements of pH value were made with a Beckman Model G pH meter and a glass electrode. Lecithinase activity was measured as previously described (Kushner, 1957).

RESULTS

Development of alkali resistance

In nutrient broth + phosphate buffer 9·5 was the highest pH value at which *Bacillus cereus* Mu-3055 grew, and there was an extended lag period before growth began (Fig. 2). The organism was induced to grow readily at pH values of 9·5 or higher by repeated subculture in media of increasing alkalinity, as follows: a 24 hr. culture of *B. cereus*, which had been maintained routinely on nutrient agar, was inoculated into a tube of nutrient broth + phosphate buffer, pH 7·4, and incubated with shaking until an optical density of about 1·0 was reached. One-half ml. of this culture was then placed in each of a series of tubes at graded pH values from 7·4 up. After shaking for 8–12 hr., the tube of highest pH value showing growth comparable to that found in the tube of pH 7·4 was selected for subculture in a series of tubes of the same and higher pH values. The process was continued, selecting each time bacteria from the tube of highest pH value showing vigorous growth. When necessary, serial subculture at the highest pH value possible was continued until the bacteria had acquired the ability to grow at a slightly higher pH value.

The course of a typical procedure is shown in Fig. 1. It is seen that the bacteria were quickly induced to grow readily at pH 9·5, but the ability to grow in media of higher pH developed much more slowly. Bacteria had to be serially subcultured several times at each pH increment before they showed any increased alkali resistance. Even with numerous subcultures the resistant *Bacillus cereus* could not be induced to grow in broth of pH value greater than 10·3.

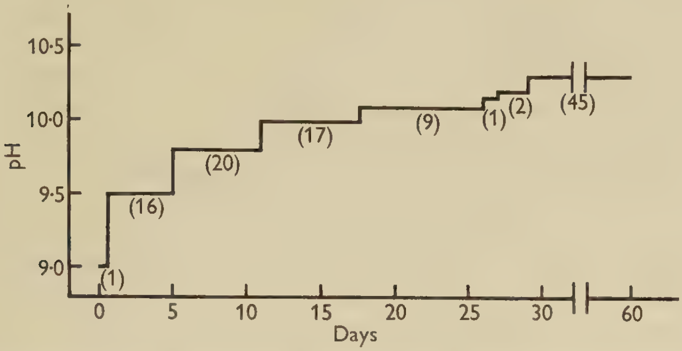


Fig. 1. Development of alkali resistance in *Bacillus cereus*. The time during which subcultures at each pH were carried out is shown on the abscissa. The actual number of subcultures at each pH is shown in parentheses in the figure.

After the bacteria had begun to grow at the higher pH values, subcultures were continued at intermediate values from pH 9·5 up. This was essential, since it was frequently found that bacteria which at first grew well at the highest pH value tested died out after several subcultures at the same pH value. In these cases, it was necessary to continue the 'inducing' process with bacteria 'established' at a slightly lower pH value.

After bacteria had succeeded in growing at pH 9·5, 10·0 and 10·3, they were spread on buffered nutrient agar plates of these pH values and maintained by transfer every few days on alkaline plates or slopes. Normal bacteria were also maintained on buffered nutrient agar at pH 7·4 and were used as 'sensitive bacteria' in subsequent experiments. As was expected, normal bacteria, unable to grow in alkaline liquid media, were also unable to grow on alkaline agar plates. Resistant bacteria have been maintained in our laboratory for periods of up to one year.

Nutrient broth + phosphate buffer was used in all cases as a medium for inducing alkali resistance in the bacteria whose properties are described here. Preliminary experiments showed that with subculture in casein hydrolysate + tris buffer medium of gradually increasing pH values alkali resistance was acquired in approximately one-third the time, though the degree of resistance reached was no higher.

Colony formation and morphology of resistant organisms

On alkaline agar the resistant cultures grew more slowly than they (or sensitive organisms) grew on neutral agar. The colonies were smaller than those of normal *Bacillus cereus* Mu-3055 grown for the same length of time, but otherwise they appeared the same. The total quantity of bacteria growing when resistant organisms were spread on an alkaline plate was relatively small. When resistant organisms were grown on nutrient agar at pH 7·4 there was no apparent difference between their growth and that of normal *B. cereus*.

Microscopically, resistant bacteria growing on alkaline agar exhibited marked morphological changes. The presence of the buffer itself caused no such change; sensitive bacteria grown on buffered nutrient agar at pH 7·4 appeared the same as those growing on unbuffered agar. The resistant bacteria growing at pH 9·5 had an appearance only slightly different from that of sensitive bacteria; the cells were a little thinner and some were longer than those of sensitive cultures. The decrease in cellular diameter was more marked in bacteria growing at pH 10·3 and the organisms differed considerably in length.

When resistant organisms from plates at pH 10·3 were subcultured at pH 7·4 the organisms appeared microscopically like normal *Bacillus cereus* and formed spores. As will be seen below, resistant organisms retained their alkali resistance when grown at pH 7·4. There is a possibility that resistance may be transmitted through the spores; this has not yet been investigated.

Growth of sensitive and resistant bacteria in alkaline liquid media

The effect of pH value on the growth of sensitive and alkali-resistant organisms in nutrient broth + phosphate buffer is shown in Fig. 2. Measurements of the maximal growth rates and final bacterial concentrations obtained in this and other similar experiments are given in Table 2. In this type of experiment, the inocula consisted of equal quantities of sensitive or resistant bacteria from agar plates (18–24 hr. culture), washed twice with distilled water before being added to broth tubes. The actual weights of bacteria added in each experiment were as given; the volume of bacterial suspension used varied with the bacterial concentrations but was in no case greater than 0·10 ml.

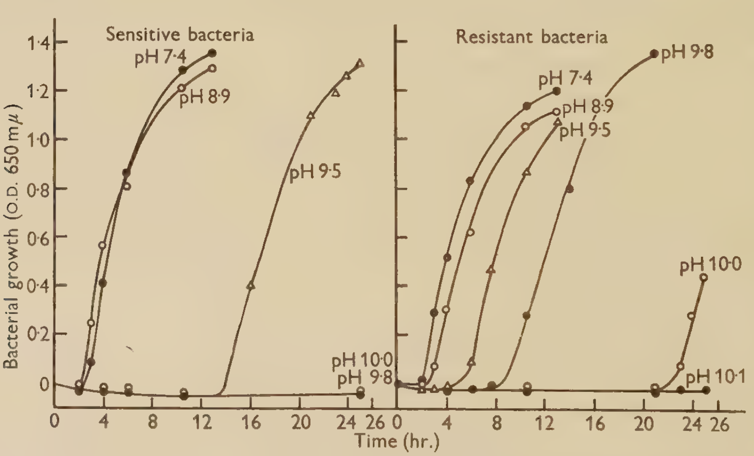


Fig. 2. The effect of pH on growth of sensitive and resistant bacteria in liquid medium. Nutrient broth + phosphate buffer was used. The initial pH of each tube is shown in the figure. The resistant bacteria had been maintained on agar of pH 10·3. The inoculum used of sensitive or resistant bacteria was 0·36 mg.

Resistant bacteria from alkaline agar plates were used in the first experiments. Once it was found, however, that resistant bacteria retained their resistance when grown on neutral medium (see below), neutral agar plates were used in certain specified experiments for preparing inocula of resistant organisms.

Differences in pH range for growth as between sensitive and resistant bacteria are immediately apparent (Fig. 2). At pH 7·4 the lag period for growth of both sensitive and resistant organisms was approximately the same and this was found in practically all experiments. The sensitive bacteria grew at pH 9·5 only after a lag period of 14 hr. and grew at no higher pH during a 25 hr. period. Sensitive bacteria did not grow at pH 10·0 in experiments lasting 30 hr. or more. The resistant bacteria, however, grew at pH 9·5 after a 4 hr. lag and grew up to pH 10·0. The resistant bacteria used in the experiment shown in Fig. 2 had originally grown at pH 10·3 in liquid media before subculture on alkaline agar. A small loss of resistance, therefore, occurred upon transfer to agar, or possibly upon washing the organisms.

Table 2. *Growth rates and final bacterial concentrations attained by sensitive and alkali-resistant bacteria in nutrient broth + phosphate buffer of different initial* pH *values*

Expt.	Bacteria	pH	Maximal growth rate (O.D./hr.)	Maximal bacterial concentration (O.D.)
1	Sensitive	7·4	0·253	—
		8·9	0·193	—
		9·5	0·140	—
	Resistant	7·4	0·183	—
		8·9	0·180	—
		9·5	0·150	—
		10·0	0·187	—
2	Sensitive	7·4	0·226	1·46
		9·3	0·210	1·24
	Resistant	7·4	0·266	1·41
		9·3	0·188	1·30
3	Resistant	7·4	0·234	1·42
		9·3	0·204	1·35
4	Sensitive	7·4	0·350	—
		9·5	0·253	—
	Resistant	7·4	0·306	—
		9·5	0·285	—

The maximal growth rate was taken as the rate of increase in optical density between O.D. 0·100–0·700.

Rates in Expt. 1 are from the experiment illustrated in Fig. 2. Figures for maximal bacterial concentration were obtained 15–20 hr. after growth began.

Weight of inocula: Expt. 1, 0·36 mg.; Expts. 2 and 3, 0·10 mg.; Expt. 4, 0·30 mg.

As indicated in Fig. 2, in media of high pH before the onset of growth there was a slight decrease in optical density. This suggests that under strongly alkaline conditions some lysis of bacteria occurred.

The measurements given in Table 2 show that, despite variations from one experiment to another, sensitive and resistant bacteria in general grew at closely comparable rates at pH 7·4. Raising the pH tended to decrease the maximal growth rate to a varying degree; but even at the highest initial pH growth, if it occurred at all, proceeded relatively rapidly. The word 'initial' is stressed because, as will be seen, bacteria growing in the buffered alkaline media used here caused a rapid decrease in the pH value. An accurate estimate of the considerable effect of pH on growth rate can only be obtained if the initial pH is held constant by frequent additions of alkali.

Little difference was noted between the maximal concentrations reached by both sensitive and resistant bacteria (Table 2). In nutrient broth + phosphate buffer maximal growth was reached at an optical density of 1·3–1·4. In the other media used there was a slightly higher maximal optical density (1·5–1·6). Maximal growth rates were similar to those found in nutrient broth + phosphate buffer.

Table 3. *Effect of* pH *on growth of alkali-sensitive and resistant bacteria in different media*

Medium	Duration of expt. (hr.)	Bacteria in inoculum (mg.)	Initial pH*	$T_{0\cdot4}$ (hr.)†		Final pH	
				Sensitive	Resistant	Sensitive	Resistant
Nutrient‡ broth + phosphate	25	0·36	7·4	4·0	3·5	7·5	7·5
			8·9	3·5	5·0	8·2	8·2
			9·5	16·0	7·2	8·3	8·3
			9·8	> 25	11·0	9·2	8·5
			10·0	> 25	24·8	9·4	8·7
			10·1	> 25	> 25	9·5	9·5
Nutrient broth + tris buffer	27	0·36	7·5	2·5	2·7	7·7	7·9
			9·2	3·2	4·0	8·6	8·7
			9·5	4·5	5·5	8·7	8·8
			9·8	9·2	6·2	8·7	8·9
			10·0 (9·5)	15·0	7·0	8·8	8·9
			10·2	22·5	10·5	9·0	9·0
			10·5 (9·6)	25·0	13·5	9·2	9·0
			10·9	> 27	19·0	9·7	8·9
			11·2 (9·9)	> 27	> 27	9·8	9·8
Casein hydrolysate + tris buffer	25	0·28	7·4	2·5	2·8	8·1	8·1
			8·9	3·2	3·4	8·7	8·6
			9·4	4·6	5·2	8·9	8·9
			9·8 (9·7)	25·0	16·0	9·7	9·2
			10·0 (9·9)	> 25	17·0	9·9	9·3
			10·3 (10·2)	> 25	> 25	10·2	10·1

Resistant bacteria as in Fig. 2; organisms growing in casein hydrolysate + tris buffer had been subcultured at pH 7·4 before use.

* Figures in parentheses show final pH after incubation in the absence of bacteria.

† Time at which cultures reach a (net) optical density of 0·4.

‡ The results given are those illustrated in Fig. 2.

In these experiments the most striking and consistent effects of the pH value of the medium were those produced on the lag period. Growth curves can conveniently be described in terms of this figure or, more accurately, in terms of the time necessary for the bacteria to reach a defined net optical density. Here, the value $T_{0\cdot4}$, that is the time at which an optical density of 0·4 is reached, is used to describe bacterial growth. The $T_{0\cdot4}$ values for the curves shown in Fig. 2 are given, with other data, in Table 3.

The pattern of growth of sensitive and resistant organisms in nutrient broth + tris buffer medium and in casein hydrolysate + tris buffer medium was similar to that found in nutrient broth + phosphate buffer (Table 3). Both sensitive and resistant organisms grew at a slightly higher initial pH range in nutrient broth + tris buffer medium than in the other two media. This was probably due to the more rapid spontaneous decrease of pH value found in the nutrient broth + tris buffer medium (Tables 1 and 3). In these experiments, sensitive bacteria were compared with bacteria maintained at pH 10·3. Resistant cultures maintained on buffered agar plates at pH values between 7·4 and 10·3 had, as was expected, intermediate resistance in liquid media. As the figures in Table 4 show, organisms from nutrient agars of progressively increasing pH values showed progressive shortening of the $T_{0\cdot 4}$ value in alkaline media. Within the weight range used in these experiments, altering the size of inoculum had little effect on the growth. It was found, in addition, that decrease of inoculum size from equivalent 0·30–0·02 mg. dry wt./tube increased the lag period and the $T_{0\cdot 4}$ value by about 25 % at all pH values tested; increase of inoculum size, up to equivalent 1 mg. dry wt./tube had relatively little effect.

Table 4. *Effect of* pH *on growth of bacteria of varying degrees of resistance in liquid medium*

pH of medium	$T_{0\cdot 4}$ (hr.) Bacteria from agar of pH 7·4	9·5	10·3
7·4	3·0	3·6	2·8
9·4	>12	7·3	5·0
9·9	>12	>12	11·0

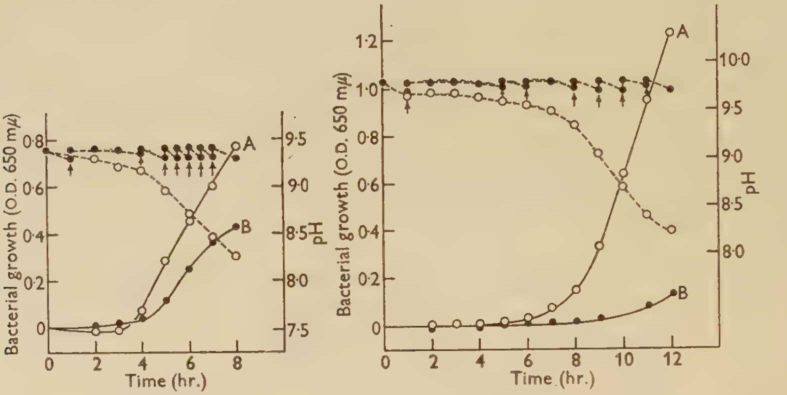
A 0·34 mg. inoculum of bacteria was used in all tubes. Growth medium was nutrient broth + phosphate buffer.

Acid production by sensitive and resistant bacteria

Both sensitive and resistant bacteria growing in media at pH 8·9 or higher caused the pH value of the medium to fall (Table 3). In nutrient broth + phosphate buffer (pH 7·4) no change in the pH value occurred with growth; in tris-buffered media (pH 7·4–7·5) growth caused a small increase in pH value. When the organisms did not grow, the pH value of alkaline media fell only to the same extent as in media to which no bacteria were added (Tables 1 and 3).

Acid production was in some cases quite vigorous; the decreases in pH value which took place indicate that the growing bacteria produced 0·1–0·3 m-equiv. acid (corresponding to 0·1–0·3 ml. N-acid). The actual acid(s) produced were not determined. Possibly, as with *Escherichia coli* (Gale, 1948), *Bacillus cereus* Mu-3055 growing in alkaline media lowered the pH by deaminating some of the amino acids present and forming carboxylic acids.

The relation between acid production and the phase of bacterial growth is of importance in considering possible mechanisms of alkali resistance. This relation was studied in the experiments illustrated in Fig. 3 in which the growth of resistant cultures and the pH changes were followed simultaneously. Before growth began the pH values of the medium declined slowly, the rate of decline being equal to the spontaneous rate already described (Table 1). After the onset of growth, the pH value fell much more rapidly and continued to fall while growth continued. When the pH of the medium was not permitted to fall freely but was held constant by periodic additions of NaOH solution, the resistant cultures still grew. The rate of growth was somewhat lower at pH 9·4, and considerably lower at pH 9·8, than in media in which the pH value was permitted to fall. Significant growth did take place when the pH was held at 9·8.



a *b*

Figs. 3 *a*, *b*. Growth of resistant bacteria in alkaline media of varying and constant pH. Solid lines represent bacterial growth, and broken lines represent the pH of the medium. Curves *A* show bacterial growth that took place when the pH fell freely, and curves *B* the growth that took place when the pH was held at the initial value by periodic additions of NaOH (indicated by arrows). *Bacillus cereus* resistant to growth on agar of pH 10·3 was subcultured at pH 7·4 before use; 1·2 mg. inocula were placed in 200 ml. lots of nutrient broth + phosphate buffer in 500 ml. Erlenmeyer flasks, incubated with shaking, and samples taken at the times indicated for pH and optical density readings. (*a*) Initial pH of 9·4. (*b*) Initial pH of 9·8.

In an experiment similar to that shown in Fig. 3*a* an inoculum of 4·2 mg. resistant organisms was used. The rate of pH change relative to growth was the same. The organisms used in the experiment shown in Fig. 3 had been subcultured once at pH 7·4. Resistant bacteria taken directly from agar of pH 10·3 caused the same rate of fall of pH when grown on liquid medium of pH 9·5.

The relation between growth phase and acid production by sensitive organisms multiplying in nutrient broth + phosphate buffer at pH 9·4 was similar to that found in the resistant cultures. The pH value remained almost constant during the prolonged lag phase and fell rapidly when growth began (Fig. 4). Comparison of Fig. 3*a* with Fig. 4 shows that the rate of pH decline caused by growing sensitive organisms was similar to that caused by growing resistant organisms. Between pH 9·0 and 8·5, when the bacteria were growing at the maximum rate, the respective rates of pH decline were identical.

Stability of alkali resistance

That alkali resistance was not lost when resistant organisms were subcultured on agar of pH 7·4 was indicated by the fact that such organisms grew when replated on alkaline agar. A more detailed experiment on this point is shown in Table 5. Resistant cultures of the organism were serially subcultured 6 times at pH 7·4, and the effect of pH on the growth of these subcultures was tested. There was no loss of alkali resistance during growth at pH 7·4. Indeed, the $T_{0\cdot4}$ values at pH 9·5 and especially at pH 10·0 for three of the four

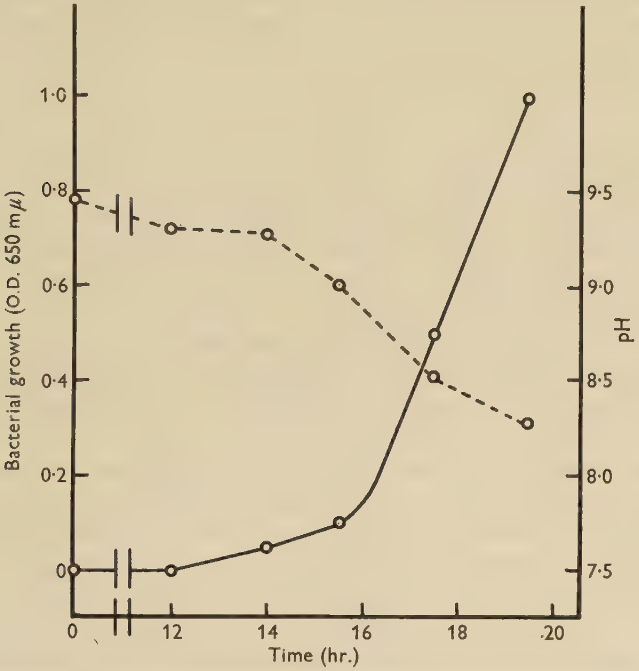


Fig. 4. Changes in pH during growth of sensitive bacteria in alkaline medium. Conditions and legend as in Fig. 3*a* except that a 4·4 mg. inoculum of sensitive organisms was used and the pH was not held constant.

Table 5. *Retention of alkali resistance during subculture on agar of* pH 7·4

		$T_{0\cdot4}$(hr.) No. of subcultures				
		0	1	2	5	6
pH	Sensitive bacteria	Resistant bacteria subcultured on agar of pH 7·4				
7·4	4·0	4·4	3·2	3·2	3·9	2·0
9·5	16·0	7·2	6·2	4·8	7·2	4·3
10·0	>25	21·5	12·0	11·2	19·0	13·0

A 0·28 mg. inoculum of bacteria was used in each experiment. Resistant *B. cereus* had been maintained previously on agar of pH 10·3, and was serially subcultured on buffered agar of pH 7·4 at 24 hr. intervals. The 5th subculture was stored overnight at 5° before testing. Growth medium for testing pH sensitivity was nutrient broth + phosphate buffer.

subcultures tested were lower than those of the parent culture. It may be significant that the one member of the series shown in Table 5 virtually indistinguishable from the parent culture had been held overnight in the cold before testing, but these findings have not been further investigated.

Lecithinase production by resistant bacteria

Because of the possibility that the lecithinase which is produced by normal *Bacillus cereus* plays a part in its pathogenicity to insects (Heimpel, 1955*b*; Kushner & Heimpel, 1957), the ability of resistant *B. cereus* to produce this enzyme was measured. The resistant organisms when grown in unbuffered 2 % (w/v) casein hydrolysate produced amounts of lecithinase comparable to those produced by a normal culture of sensitive *B. cereus*.

DISCUSSION

In considering alkali resistance it is natural to refer to the work already carried out on the nature and development of drug resistance in bacteria (reviewed, for example, by Bryson & Szybalski, 1955; Davis, 1957; Schnitzer & Grunberg, 1957). However, two differences must be noted between drug resistance and the resistance to alkaline pH values described here. First, with alkali resistance one is not dealing with a chemical compound foreign to the cell but simply with an excess concentration of hydroxyl ion. Secondly, a drug may affect or be thought to affect only one enzyme, but pH changes might affect the action of many enzymes.

Since such a general inhibitor as [OH] is involved we may, at the start, rule out two theoretical mechanisms of resistance. Neither the formation of some metabolite in sufficient concentrations to overcome a specific metabolic block produced by a high pH value, nor the production of alternative metabolic pathways to circumvent such a block appears to be likely. The role that acid production by the growing bacteria plays in overcoming the toxic effects of high pH values deserves special consideration, and it seems important to distinguish between what may be termed primary and secondary mechanisms of resistance. If acid production were completely responsible for resistance, then we would expect the resistant inoculum to lower the pH value of the medium before growth began. This, however, did not occur. Except for a slight fall, found even in the absence of bacteria, the decrease of pH value accompanied, but did not precede, growth. When the pH value was maintained by periodic additions of alkali, growth still took place, though at a lower rate than in media of decreasing pH value. It should be noted that sensitive bacteria may grow in media of pH 9·4–9·5, though after a much longer lag period than resistant bacteria, and that during growth their rate of acid production is much the same as that of the growing resistant bacteria. Resistant bacteria, then, are distinguished from sensitive bacteria not by their ability to produce acid during growth in alkaline media but by their greater ability to begin to grow in these media. Acid production by the growing bacteria may be regarded as a secondary mechanism of resistance, which increases the rate of growth once this has begun. It is this acid production that appears responsible for the fact that growth curves in media of initially different pH values differ mainly only in the length of the lag period.

Different possible primary mechanisms of resistance, which might enable resistant organisms to begin growing in highly alkaline media, have been considered. One possibility is that the resistant organisms have a limited permeability to hydroxyl ion or have an active mechanism that regulates the passage of hydroxyl ion into the cell. If either of these mechanisms were in operation, then a marked difference between intra- and extracellular pH might occur. In this case, individual enzymes in intact resistant cells might be less affected by external alkaline conditions than the same enzymes in intact sensitive cells. This possibility should be open to direct investigation.

Another hypothetical possibility is that of a shift in the pH–activity curves of several enzymes in the resistant cell. Kocholaty & Weil (1938) and Kocholaty & Hoogerheide (1938) noted a shift toward the acid side of extracellular proteinase produced by *Clostridium histolyticum* and of several dehydrogenases in *C. sporogenes* as these organisms were grown in more acid media. A similar shift in pH–activity curves towards the alkaline side is conceivable in alkali-resistant *Bacillus cereus*.

The detailed genetic aspects of alkali resistance have not yet been studied. Resistance appears to be a heritable property, transmitted from subculture to subculture, and persisting in neutral media. Its slow and gradual development suggests that resistance to the highest alkalinities does not arise through selection of a few resistant organisms originally present in the sensitive population, nor through a single-step mutation. A series of mutations, each leading to slightly increased resistance, might be involved. As Bryson & Szybalski (1955) pointed out: 'In a strict sense, proof that clones of drug-resistant cells depend in origin upon mutation can be supported only by genetic tests, for example, study of bacterial recombination, transduction, transformation, or analysis of the segregation of nuclear hereditary determinants.' This statement must apply as well to any genetic interpretation of our results.

We are indebted to Dr S. G. Smith for first suggesting this line of approach to the problem of increasing pathogenicity. Dr T. A. Angus provided invaluable assistance in examining our bacteria and in discussing changes in microscopic and colonial morphology. We have to thank Dr A. M. Heimpel for valuable discussions.

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Chromatin Patterns in *Spirillum anulus*

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SUMMARY: The chromatin configurations of acid-Giemsa preparations of *Spirillum anulus* were followed sequentially, at 2 hr. intervals, for 48 hr. A series of broth cultures were inoculated with 0·1 ml. of a 7-day broth culture, incubated at 30° and centrifuged at 2 hr. intervals. The sedimented cells were used to inoculate agar plates for use in making smears by the agar block method. The smears were fixed in acid-alcohol, hydrolysed in N-HCl solution at 60° for 8 or 15 min. and stained with Giemsa. Samples of living cells, taken from the sedimented cells at all time intervals, were examined by phase-contrast and darkfield microscopy.

The majority of the early cells (0–16 hr.) were resistant to acid hydrolysis, being undifferentiated by 15 min. of hydrolysis until the 12th hr. of growth. After the 16th hr. of the growth cycle the cells showed an abrupt change in their reaction toward acid hydrolysis; subsequent preparations were hydrolysed for 8 min. The early cells appeared undifferentiated by phase contrast until approximately the 14th hr. of growth; after this time inclusions could be observed in the cells. Transparent cells containing dark inclusions were observed in the living cells by the 16 hr. of growth and coincided with the abrupt change of the acid-Giemsa cells toward acid hydrolysis.

The pattern of the chromatin configurations found in the acid-Giemsa cells consisted in : (1) an axial filament which fragmented into thick bars, from which spherical bodies were formed; (2) the direct division of the spherical bodies into smaller spheres, from which bead-like granules were formed by means of intermediate X-, Y-, and V-forms or the direct formation of rings of bead-like granules from the spherical bodies; (3) the re-formation of the axial filaments from the bead-like granules with the aggregation of all the chromatin material of the cell at the centre of the cells; (4) the division of the aggregated chromatin material on cell division and the extension of the chromatin material in the form of an axial filament; and (5) the re-formation of the spherical bodies and bead-like granules as outlined in steps (1) and (2) from the axial filaments.

Essentially the same types of chromatin configurations were observed in living cells. The most exact correlation between the living and fixed images of the chromatin bodies of *Spirillum anulus* occurred between the 18th and 30th hr. of the growth cycle. The correlation between the living and fixed images of the chromatin bodies of *S. anulus* was less exact at other time intervals. Various theories concerned with the occurrence of axial filaments and chromatin aggregations in bacterial cells are discussed.

Robinow (1956*a*, *b*) has pointed out that much of the confusion which has arisen in the interpretation of chromatin bodies of bacteria is due to the use of bacterial cells of differing ages of growth. He has called attention to the fact that the 'nucleoids and chromatinic dumb-bell bodies', shown in bacterial cells by certain cytologists (Badian, 1933; Stille, 1937; Robinow, 1944), were demonstrated in cells from 'very young growing cultures' whereas the chromidial nuclei and axial filaments of the older school of bacterial cytologists

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(Guilliermond, 1908; Dobell, 1911; Delaporte, 1939, 1950) were found in cells from older cultures.

Robinow (1956*b*) has also called attention to the discrepancy between the living and fixed images of the chromatin bodies of bacterial cells. Studies of direct observation of the chromatin bodies of living cells have been made by a number of investigators, among whom are Stempen (1950), Clifton & Ehrhard (1952), and Mason & Powelson (1956). With the exception of the article by Mason & Powelson (1956) the information to be gained about the morphology and division of the chromatin bodies of living bacterial cells has been rather meagre, consisting mainly in the information that the light areas, as observed by phase contrast, are correlated with the position of the chromatin bodies in fixed, hydrolysed and stained cells. Mason & Powelson (1956) were able to correlate karyokinesis with cytokinesis in living cells of *Escherichia coli*, but their findings in *Bacillus cereus* were less conclusive.

The chromatin bodies of *Spirillum anulus* have been followed in acid-Giemsa and living cells from resting stage (non-dividing cells) to resting stage in an attempt to resolve some of the problems of the chromatin bodies of bacteria, as pointed out by Robinow (1956*a*, *b*).

METHODS

Cultural methods. Spirillum anulus was grown in broth of the following composition: peptone, 5·0 g.; beef extract, 3·0 g.; yeast autolysate, 3·0 g.; distilled water, 1 l. A solid medium was prepared by adding 16·5 g. agar/l. to the above formula. To insure a plentiful supply of resting cells (non-dividing cells), 3 broth tubes were inoculated with 0·1 ml. of sedimented cells of *S. anulus* on each of 7 days prior to the beginning of the experimental procedures. A series of broth tubes was inoculated with 0·1 ml. of a 7-day broth culture, using 6 broth tubes at each time interval. The times of inoculation were varied so that cells in consecutive stages of growth could be obtained during one working day. The subcultures were incubated at 30° and centrifuged 2 hr. before use. The supernatant fluid was discarded by decanting and the tubes shaken vigorously to redistribute the cells in the small amount of fluid left (*c.* 0·2 ml.). A sample was removed, at each time interval, from the sedimented cells for the examination of living cells by phase contrast and darkfield microscopy. Three agar plates were inoculated with 0·1 ml. of the sedimented cells at 2 hr. intervals. The plates were incubated at 30° for at least 2 hr. before use; they were not used after 6 hr. incubation.

Fixed cells. Impression smears were made by the agar block method on glass slides, 6 slides being made at each time interval. The smears were allowed to come just to dryness and placed in acid-alcohol fixative (1 part glacial acetic acid to 3 parts ethanol) for 10 min. The fixed smears were passed through a graded series of ethanol and distilled water to distilled water and hydrolysed in N-HCl solution at 60°. Early cells (0–16 hr.) were hydrolysed for 15 min.; subsequent preparations were hydrolysed for 8 min. After hydrolysis the smears were rinsed in distilled water and stained in Giemsa solution, pH 7·0, for 30 min.

Living cells. At each 2 hr. interval, one drop of the sample of the living cells was placed in a drop of broth on a sterile glass slide, covered with a sterile no. 0 coverslip and excess fluid was removed with filter-paper. The preparations were ringed with immersion oil to prevent drying and observed by phase-contrast and darkfield microscopy.

Photography. The stained smears were examined with the Leitz Ortholux microscope, equipped with an achromatic condenser, an apochromatic objective (×90), N.A. 1·25 and examined with compensating oculars (×12). The photographs were made with the Leitz Aristophot camera using Panatomic X sheet film and a 560 mμ interference filter.

Observations and photographs of living cells were made with the phase attachment of the Leitz Ortholux microscope which permits a continuous transition from 'brightfield' to 'phase contrast' to 'darkfield' by the adjustment of the height of the mirror body in the condenser. An apochromatic oil-immersion phase-contrast objective (×90) N.A. 1·10 and compensating oculars (×12) were used for photographs and observations. The photographs were made with the Leitz Makam camera, using Royal Pan sheet film and employing the green filter supplied with the Leitz phase equipment.

RESULTS

There were two reasons for the use of *Spirillum anulus* as the experimental organism, the more important of these being the slow growth rate which permitted the comparison of the chromatin configurations of acid-Giemsa cells with those of the living cells of the same age. The large size of the cells which provided chromatin configurations of considerable size was of secondary importance.

Although the cells of *Spirillum anulus* were followed for a 7-day period (the time necessary to proceed from resting cells to resting cells), the results reported are those found for the first 48 hr. only. The essential pattern of the chromatin configurations was established within this time limit, and the configurations observed on the 3rd and 4th days of the growth cycle were essentially the same as those to be reported for the 2nd day. All types of chromatin configurations were found by the 5th day of growth, and by the 6th day the cells showed only sporadic division. The cells were in the resting phase of the growth cycle by the 7th day.

Previous investigations of the species of *Spirillum* (Williams, 1955; Williams & Rittenberg, 1957) have shown that the chromatin configurations of acid-Giemsa cells are contained within or associated with the large inclusions of the cells. The large inclusions, however, show staining reactions considered to be diagnostic of volutin by Grimme (1902). Despite this fact, data will be presented to show that the inclusions of the living cells may be considered to be the equivalent of the chromatin bodies of the acid-Giemsa cells. Although the large inclusions of the unhydrolysed cells of *Spirillum anulus* show staining reactions of volutin, this is not true for the small inclusions.

No effort was made to obtain synchronized cultures of *Spirillum anulus*.

A heterogeneous population was observed in the very early cultures (0–10 hr.), as well as in cultures after the 5th day of the growth cycle. Between the 16th and 48th hr. of growth, the cells showed essentially the same type of inclusion in the living cells, at a particular time interval, and the chromatin configurations found in acid-Giemsa cells, at a particular time interval, were similar.

Dividing cells were not observed in the living cells prior to the 6th hr. of growth and then cell division was observed only sporadically. After the 14th hr. of growth, the number of dividing cells increased but was still small. The greatest proportion of dividing cells was observed between the 30th and 48th hr., at which time approximately 50 % of the cells were either dividing or showed evidence of recent cell division. The criterion of cell division was constriction and fission; that of recently divided cells was the short $\frac{1}{2}$ to $1\frac{1}{2}$ curved forms shown in Pl. 1, figs. 6–9. The correlation between karyokinesis and cytokinesis, found by Mason & Powelson (1956) for living cells of *Escherichia coli* was anticipated but not observed. The chromatin bodies of living cells of *Spirillum anulus*, as observed by phase-contrast or darkfield microscopy, resembled the forms shown by Mason & Powelson (1956) for *Bacillus cereus* rather than those of *E. coli*.

The data reported for living and fixed cells of *Spirillum anulus* are divided into three sections: (1) early cells (0–16 hr.) in which cell division was observed to occur only sporadically; (2) young cells (16–30 hr.) in which cell division occurred in an increasing number of the cells; and (3) mature cells (30–48 hr.) during which time the greatest proportion of dividing cells was observed.

Early cells (0–16 *hr.*)

Acid-Giemsa cells. The appearance of approximately 90 % of the cells, prior to the 12th hr. of growth, was undifferentiated (Pl. 1, figs. 1–3). These cells were extremely resistant to acid hydrolysis, appearing undifferentiated even when hydrolysed for 30 min. Since the prolonged hydrolysis time did not reveal discrete chromatin bodies within the cells, a 15 min. period of hydrolysis was used for such preparations. The reaction of the cells toward acid hydrolysis changed abruptly after the 16th hr. of growth and subsequent preparations were hydrolysed for 8 min only.

The chromatin material of the cells of *Spirillum anulus* could be differentiated from the intensely stained cytoplasm by the 12th hr. (Pl. 1, fig. 10). At this time the chromatin material appeared as an axial filament which fragmented into thick bars by the 14th hr. of growth (Pl. 1, fig. 11). Spherical chromatin bodies were formed from the bars (Pl. 1, fig. 12) by the 16th hr. These spherical bodies either divided directly (Pl. 1, fig. 14) or formed curved bar-like structures (Pl. 1, fig. 13).

Living cells. The majority of the living cells appeared undifferentiated until the 14th hr. of growth (Pl. 1, figs. 4–5). A few of the cells (approximately 10 %) contained large inclusions which subsequent observations showed to be similar to those observed in living cells on the 4th and 5th days of the growth cycle. Since no effort was made to obtain synchronized cultures, a heterogeneous population was not unexpected. The visual appearance of the early cells, by phase contrast, was that of purplish cells as noted by Clifton & Ehrhard (1952) for cells of *Bacillus anthracis*.

Inclusions could be observed in a small percentage of the cells by the 14th hr. (Pl. 1, fig. 15), but the majority of the cells remained undifferentiated (Pl. 1, fig. 9). The number of the inclusions in the cells increased by the 16th hr. but it was difficult to differentiate the periphery of the inclusions from the dark cytoplasm in phase-contrast observations (Pl. 1, fig. 16*a*), the inclusions appeared as bright purplish bodies in a dull purple cytoplasm and they photographed as empty spaces in the phase-contrast photographs (Pl. 1, fig. 16*a*). Darkfield observations and photographs, however, showed the spherical shape of the inclusions (Pl. 1, fig. 16*b*).

Young cells (16–30 *hr.*)

Acid-Giemsa cells. The changes in the chromatin configurations of the cells of *Spirillum anulus* occurred more rapidly after the 16th hr. of the growth cycle. Between the 18th and 20th hr. the chromatin bodies appeared as transverse bar-like structures (Pl. 2, figs. 19–20). By the 22nd hr. solid or broken ring-like structures, surrounding an achromatic core, appeared within the cells (Pl. 2, fig. 21), but these forms were observed with greater frequency by the 24th hr. (Pl. 2, fig. 22). Both types of configurations were often observed within the same cell (Pl. 2, fig. 23). The ring-like structures appeared to break up into smaller bodies and form small bead-like granules by means of intermediate X-, Y- and V-forms (Pl. 2, figs. 21–23). The bead-like granules predominated in the cells by the 24th hr. of growth, either as discrete bodies or as rings or bars of interconnected granules (Pl. 2, figs. 22, 30). Various arrangements of the bead-like chromatin granules were observed in the cells until approximately the 30th hr. of growth.

Living cells. The cytoplasm of the cells became increasingly lighter after the 16th hr. of growth and the inclusions could be observed with greater accuracy (Pl. 1, figs. 17, 18). By the 20th hr. the majority of the cells appeared transparent with dark purple inclusions (Pl. 2, figs. 27, 29, 31–35). The appearance of the transparent cells in the cultures of *Spirillum anulus* coincided with the change in the reaction of the fixed cells toward acid hydrolysis. Transparent cells were the only ones observed between the 20th and 24th hr. of the growth cycle. Transparent cells were observed by Clifton & Ehrhard (1952) in phase-contrast observations of living cells of *Bacillus anthracis*, but these authors considered such cells to be non-viable. The transparent cells of *Spirillum anulus*, however, were viable and growth and cell division occurred normally in such cells (Pl. 2, fig. 27). The cytoplasm of a few cells had resumed the dark appearance by the 24th hr. and was dark in the majority by the 30th hr.

The morphology of the inclusions during the transparent phase of the cells was varied. Both large and small inclusions (Pl. 2, figs. 24*b*, 31) were common between the 18th and 20th hr., although the transverse bar-like forms were also observed at this time (Pl. 2, fig. 25*b*). The predominating form of the inclusions after the 24th hr. was either the bead-like granule or ring forms (Pl. 2, figs. 27, 29, 34 and 35). The bead-like granules occurred either as discrete bodies (Pl. 2, fig. 35) or in the form of rings of bead-like granules (Pl. 2, fig. 29). The most exact correlation between the living and fixed images of the chromatin bodies of *Spirillum anulus* occurred during the transparent phase. Chromatin configurations which have a similar form in both living and acid-Giemsa cells are shown in pairs of photographs in Pl. 2, figs. 24 and 25. In these photographs, the acid-Giemsa cells are denoted by the letter '*a*', the living cells by the letter '*b*'. In figs. 24*a*, *b* the chromatin bodies are seen as spheres; in figs. 25*a*, *b* as chromatin bars. The apparent similarity between the ring-like bodies of the cells shown in Pl. 2, figs. 26*a*, *b* is a photographic illusion since the large inclusions of the living cell in fig. 26*b* had the appearance, visually, of spherical bodies consisting of a ring of dark material surrounding a brightly refractile centre. The photographic appearance of these large inclusions as rings of dark material enclosing an empty space is most probably due to the inability of black and white film to discern both colour and brightness, since such film records brightness alone (Miller & Brumitt, 1945).

Murray (1953), who also noted differences in the appearance of living and fixed chromatin bodies in bacteria, attributed these differences to the type of fixation. To determine if fixation in acid-alcohol was a source of the discrepancy between the visual appearance of these inclusions and their actual appearance in acid-Giemsa preparations, comparisons were made of the same cells in the living conditions and as fixed cells. Living cells from a 24 hr. culture are shown in Pl. 2, fig. 27, in which the large inclusions are shown in the dividing cells at the top of the photograph. In preparations fixed in acid-alcohol, however, these inclusions had the appearance of dull white, fibrous spherical bodies without the refractile centre observed in the visual observations of living cells by phase contrast. Cells from the same preparation, when fixed in acid-alcohol and stained with Giemsa for photographic purposes, are shown in Pl. 2, fig. 28. In this photograph, the large spherical inclusions which photograph as ring-like bodies (Pl. 2, fig. 27), can be seen to have a spherical form (Pl. 2, fig. 28, upper cells, marked with an arrow). Similar preparations, when fixed and hydrolysed and stained with Giemsa, showed these inclusions as ring-like structures (Pl. 2, fig. 26*a*).

No discrepancy was observed in the visual appearance of either the small spherical inclusions or the bead-like granules in any type of observation. Their morphology was essentially the same in visual observations; in their photographic appearance (Pl. 2, figs. 24*b*, 27, lower cells); in the fixed but unhydrolysed Giemsa-stained preparations (Pl. 2, fig. 28, lower cells); and in acid-Giemsa preparations (Pl. 2, figs. 24*a*, 30).

Robinow (1956*a*) has described similar ring-forms in acid-Giemsa preparations of *Bacillus megaterium* and *B. cereus*, as follows:

'...Recently divided ('resting') chromatin bodies in Giemsa preparations appear to consist only of chromatin matter, but division figures frequently appear to enclose a core of non-chromatinic matter more refractile than ordinary cytoplasm. The sudden emergence of this material during division makes one wonder whether it is not in the chromatin bodies at all times but usually invisible because we see them mostly in profile. The non-chromatinic centre of the "small *c*"-type of "resting" chromatin body, mentioned above, may well be the origin of the "core" in division figures.'

Robinow (1956*a*) considered the ring-like forms as division figures and this concept appears applicable to some of the division forms observed in living cells of *Spirillum anulus.* These forms were carefully observed during the transparent phase of the cells when they could be well differentiated from the cytoplasm. The division processes apparently consisted of two types, neither of which occurred in a clear-cut manner. One type consisted of the division of spherical inclusions (Pl. 2, fig. 31) into two smaller spherical bodies (Pl. 2, fig. 32, upper inclusion). The separation of the two halves, however, was never observed; instead these two bodies appeared to subdivide further (Pl. 2, fig. 32 (lower inclusions)), forming a semicircular body (Pl. 2, fig. 33). The semicircular bodies either formed the bead-like granules directly (Pl. 2, fig. 34) as V-shaped interconnected granules or the bead-like granules were given off from the semicircular forms by a process resembling budding (Pl. 2, fig. 34, inclusions indicated by an arrow). The other type of the formation of the bead-like granules from the large spherical bodies was by the direct formation of rings of the bead-like granules (Pl. 2, fig. 29, indicated by an arrow). The predominating form of the inclusions in the living cells between the 24th and 29th hr. of the growth cycle was that of the bead-like granule.

Mature cells (30–48 *hr.*)

Acid-Giemsa cells. By the 30th hr. of growth, the bead-like granules began to coalesce in the cells (Pl. 3, fig. 36) and, by the 32nd hr. the chromatin material appeared around the edge of the vacuoles (Pl. 3, fig. 37). Thick rings of chromatin material were observed in the cells by the 34th hr. (Pl. 3, fig. 38) with the condensation of the chromatin material at the centre of the cells by the 36th hr. (Pl. 3, fig. 39). By the 40th hr. of growth all the chromatin material was aggregated into a solidly staining mass at the centre of the cells (Pl. 3, figs. 40, 41) which divided on cell division (Pl. 3, figs. 42, 43). After cell division the compact mass of chromatin material was redistributed in the cells, along the vacuoles, in the form of an axial filament (Pl. 3, fig. 44). This separated into the curved bars and small spherical bodies (Pl. 3, fig. 45) in a manner similar to that shown in the early cells (Pl. 1, figs. 13, 14) or divided into the small spherical bodies directly (Pl. 3, fig. 46). By the 48th hr. of the growth cycle, the predominating form of the chromatin material in the cells was again the small bead-like granule (Pl. 3, fig. 48).

The chromatin configurations, found in the cells of *Spirillum anulus* between the 24th and 48th hr. of the growth cycle, were repeated, in a cyclical manner, for the succeeding 2 days (3rd and 4th days) of the growth cycle. By the 5th day all types of chromatin configurations were observed with the type shown in Pl. 3, fig. 38, predominating. By the 6th day the undifferentiated cell similar to those of the early cells (Pl. 1, figs. 1–10) were observed, and by the 7th day the undifferentiated cells predominated.

The essential pattern of the chromatin configurations in acid-Giemsa cells of *Spirillum anulus* appears to consist in: (1) the formation of an axial filament, which fragments into bars from which spherical bodies are derived; (2) the division of the spherical bodies into transverse bars and bead-like granules; (3) the condensation of the bead-like granules with the re-formation of the axial filament along the vacuoles of the cells; (4) the aggregation of all the chromatin material of the cells at the centre in a solid staining mass; (5) the division of the solidly staining mass on cell division; and (6) the re-distribution of the chromatin material in the form of an axial filament from which the bead-like granules are again re-formed. The times at which these various forms are found in the cells appears to be correlated with the age of the cells rather than with cell division.

Living cells. Observations of living cells by either phase-contrast or dark-field microscopy became increasingly difficult after the 30th hr. of the growth cycle. The dark appearance of the cytoplasm made it difficult to differentiate the periphery of the inclusions by phase contrast and the bright glare from the centre of the inclusions made darkfield observations practically impossible. A few cells could be observed clearly, at each time interval, and these appeared to be representative of the majority of the cells.

The number of inclusions was greatly increased and they were closely spaced within the cells, either as clumps (Pl. 3, fig. 49) or singly in the cells (Pl. 3, fig. 52). The clumping of the inclusions in masses or as closely adjacent bodies increased the difficulties of observation. Even when the inclusions appeared as discrete bodies (Pl. 3, fig. 53) it was often difficult to show the discrete form of the dark and brightly refractile inclusions as the edges of the two bodies often appeared to merge in the photographs. This was especially true of phase-contrast photographs (Pl. 3, fig. 50*a*); darkfield photographs, however, showed these bodies to be discrete (Pl. 3, fig. 50*b*).

The correlation between the living and fixed images of the chromatin bodies of *Spirillum anulus*, which was present during the preceding growth phase (18–24 hr.), was not so apparent at this time (30–48 hr.). No structures resembling the axial filaments or chromatin aggregations of the acid-Giemsa cells (Pl. 3, figs. 39–44) were observed in the living cells. Certain formations, however, were observed in the living cells which could have possibly given rise to the formations in the acid-Giemsa cells. The clumps of inclusions shown in Pl. 3, figs. 49, 50*a*, *b*, could have given rise to the chromatin aggregations and the closely spaced inclusions, shown in Pl. 3, figs. 51*a*, *b*, 52, could have given rise to the axial filaments.

To explain the formation of the chromatin aggregations and axial filaments from these formations of the inclusions of the living cells, however, certain assumptions, which have not been verified experimentally, would have to be made. One such assumption is that the inclusions are hollow spheres which contain a material other than chromatin which is removed by acid hydrolysis, causing the collapse of the walls of the hollow sphere. This collapse might, conceivably, give the ring-like formations which are observed in the acid-Giemsa cells. The brightly refractile centres of some of the inclusions of the living cells make this postulated structure feasible. Some corroboration for the hollow sphere structure is found in an ultrathin section of another species, *Spirillum serpens*, by Chapman & Kroll (1957). Their fig. 8 shows a cell which contains two large vacuoles, the upper of which shows a smaller sphere of electron dense material. On the other hand, if the inclusions are, in reality, disk-shaped, as postulated by Robinow (1956*a*) for similar ring-like formations in acid-Giemsa cells of *Bacillus megaterium* and *B. cereus*, the entire spherical body would have to be enclosed in a delicate membrane which is destroyed by acid hydrolysis. The premise of a membrane surrounding the inclusions is based on the findings that the unhydrolysed inclusions appear as spherical bodies (Pl. 2, fig. 28) and the necessity of such a membrane to retain the material removed by acid hydrolysis at the centre of the inclusions.

Williams (1955), in an attempt to explain the staining peculiarities of these large inclusions, has postulated that they are composites of ribose- and deoxyribose nucleic acids. This is especially true since volutin has been considered to be a type of ribose nucleic acid by Meyer (1904), Casperson & Brand (1941) and van den Berghe (1942). Knaysi (1951) states that volutin is removed from bacterial cells by acid hydrolysis and the removal of this substance from either the hollow spheres or the disk forms could account for the ring-like appearance of the inclusions (Pl. 2, fig. 26*b*). The massing of the inclusions in the cells during the growth cycle between the 30th and 48th hr. would then account for the appearance of the chromatin aggregations and the axial filaments. As was pointed out above, both the structure and the supposed arrangements which could give rise to the formations in the acid-Giemsa cells are purely speculative. One contra-indication for this supposed type of structure of the inclusions is the data of Jacobson & Webb (1952) in regard to the staining reactions of DNA and RNA in Giemsa-stained cells. These authors state that DNA alone appeared a shade of purple-red and that composites of DNA and RNA have a blue-black appearance in the chromosomes of bone marrow cells. The colour of the hydrolysed chromatin bodies in cells of *Spirillum anulus* is a bright red shade; that of the unhydrolysed chromatin bodies is a dark purple when stained with Giemsa.

Another possibility has been suggested by Robinow (1956*b*), after a consideration of the data of Mudd & Smith (1950) that hydrolysed chromatin bodies of bacterial cells have a greater electron density than unhydrolysed ones. Robinow has suggested 'the disquieting possibility that hydrolysis may cause material to shift from the cytoplasm to the nuclei.' It is possible that this shift of material from the cytoplasm to the chromatin bodies of *Spirillum anulus* may be the cause of the appearance of the axial filaments and chromatin aggregation. If so, this material cannot be RNA since the staining reaction of these bodies in the hydrolysed cells is a bright shade of red and not blue-black.

DISCUSSION

Robinow (1956*b*) has divided the chromatin bodies of bacteria into two categories—single solid shapes and open complex ones, which are described as follows: '...The solid shapes are straight or tapering bars or dumb-bells, the open ones are clusters of three or four, probably connected, round granules of the same size as the halves of the dumb-bells with one end nearly doubled, V-forms, paired dumb-bells, H- and butterfly shapes.' Some of the chromatin configurations which occur in acid-Giemsa cells of *Spirillum anulus* conform to this description by Robinow (Pl. 1, figs. 13, 14; Pl. 2, figs. 19–33). Robinow (1956*a*, *b*) has expounded on these particular types of chromatin bodies in bacteria and further comment would be superfluous. The other types of chromatin configurations found in acid-Giemsa cells of *Spirillum anulus* may be considered to be the type called 'axial filaments or cords' by Delaporte (1939, 1950) and compact aggregates by Whitfield & Murray (1954, 1956).

These particular types of chromatin configurations have been demonstrated in cells of many bacterial species by numerous investigators (Delaporte, 1939, 1950; Bisset, 1956; Whitfield & Murray, 1954, 1956). Two theories are current in regard to the occurrence of these axial filaments and chromatin aggregations. The one adopted by Delaporte (1939, 1950) and Bisset (1956) is that such configurations occur normally in bacterial cells at some time during the growth cycle. The other theory is that such configurations are a response to abnormal conditions of growth in which the metabolism of the cell is disrupted by a variety of causes. Some of the conditions under which axial filaments or chromatin aggregations have been found in bacterial cells are: (1) ageing of the cells (Kellenberger, 1953); and (2) a response to conditions deleterious to cell growth and metabolism such as exposure to bacteriophages (Whitfield & Murray, 1954), antibiotics (DeLamater, 1953; Kellenberger, 1953), high salt concentrations (Whitfield & Murray, 1956), low temperatures (Hunter-Szybalska, Szybalski & DeLamater, 1956), ultraviolet irradiation (Hartman, Payne & Mudd, 1955) as well as to other causes.

Whitfield & Murray (1954, 1956) have recently investigated the occurrence of such axial filaments and chromatin aggregations in a variety of bacterial species and have reached the conclusion that such formations are the result of an ionic imbalance within the cells due to external conditions. The experimental conditions cited by Whitfield & Murray (1954, 1956) are incontrovertible. The explanation advanced by these authors, however, does not appear to fit the data found for cells of *Spirillum anulus* under the experimental conditions cited. External conditions deleterious to growth and metabolism were not imposed. These formations were found in cells of *S. anulus* taken from cultures grown under normal conditions of medium and temperature. These forms occur in very young cultures (12 hr.), during the logarithmic growth phase (36–40 hr.) as well as in ageing cells (96–120 hr.). Exhaustion of nutrients in the broth medium can also be ruled out as a precipitating cause since they were found in cells which had been centrifuged and plated out on freshly poured agar plates and allowed to grow for at least 2 hr. before use. Whitfield & Murray (1956) have shown that such aggregations, caused by conditions deleterious to growth and metabolism, were dispersed when cells of various bacterial species, grown under abnormal conditions, were transferred to fresh normal media. Both the axial filaments and chromatin aggregations were repeated in the cells at specific time intervals and other types of configurations often intervened between the times when these forms were found.

The theory that an ionic imbalance is the cause of the aggregations of the chromatin material in the cells is less easy to refute. It appears doubtful, however, that the normal medium, in which maximum growth occurred, could be the source of an ionic imbalance within the cells. If such be the case, then the cells of *Spirillum anulus* differ drastically from other bacterial cells since the majority of the cells, at a particular time interval, showed these formations. If the medium was the source of the ionic imbalance then the same medium which precipitated the imbalance also allowed the recovery of the cells, since these forms were always followed by the bars and bead-like granules considered the normal form of chromatin bodies in bacterial cells. Cessation of growth may also be eliminated as the precipitating cause of the axial filaments and chromatin aggregations since the greatest proportion of dividing cells were observed in the living cells during the time when these configurations were the most obvious (30–44 hr.). These objections do not preclude the experimental data of Whitfield & Murray (1954, 1956); rather they call for another explanation of these phenomena.

Bisset (1956, p. 8) considers such chromatin aggregations to be compound structures similar to the meganucleus of some of the protista and compares the two types of structures as follows: '...The comparable condition in bacteria I believe to be the longitudinal rod (Pl. 1, fig. 6) which occurs in the later stages of vegetative cultures of many types of bacteria.... And the most probable explanation is that it resembles the protozoan meganucleus in being not one but many nuclei, produced by repeated nuclear divisions in anticipation of cell division.'

Compound nuclear components are not unknown in higher organisms, the best known example probably being that of *Ascaris megalocephala bivalens*, in whose gametic cells two rod-shaped chromosomes are found. Walton (1924) has shown that these two rod-shaped chromosomes break up into approximately 22 small chromosomes for the autosomes and 8 for the sex chromosomes in the somatic cells of this organism.

One explanation for the occurrence of such aggregations of the chromatin material in bacterial cells, when exposed to conditions deleterious to growth and metabolism, has not been considered. If such aggregations represent a normal procedure of the chromatin material, involved in some process of cell preservation, the exposure of bacterial cells to conditions inimical to growth and metabolism would most probably invoke this protective mechanism and thus aid in conserving the cells.

Delaporte (1939, 1950) showed axial filaments and chromatin aggregations in bacterial cells and considered them to be a normal formation in rod-shaped bacteria. Bisset (1956) considered them to be compound structures and has devised a scheme by which these compound structures function as an autogamous process. The data found for cells of *Spirillum anulus* confirm and support the theory of Delaporte (1939, 1950); the data neither confirm nor refute the theory of Bisset (1956).

I wish to acknowledge my indebtedness to Dr T. T. Chen, Department of Biology, for many helpful suggestions in regard to cytological procedures and for the stimulating discussions of the findings. I wish to express my appreciation of the technical assistance of Mr Richard Fred.

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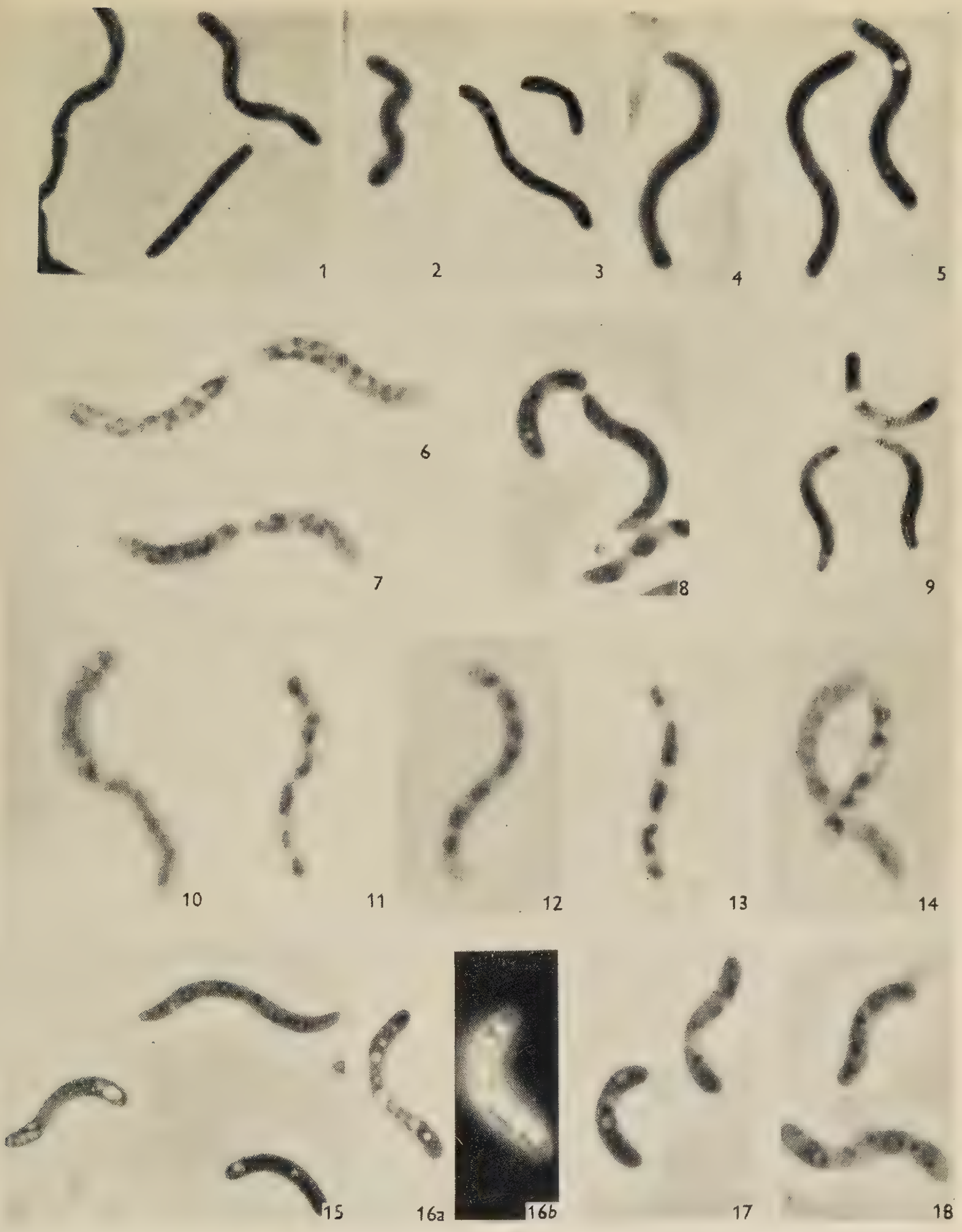
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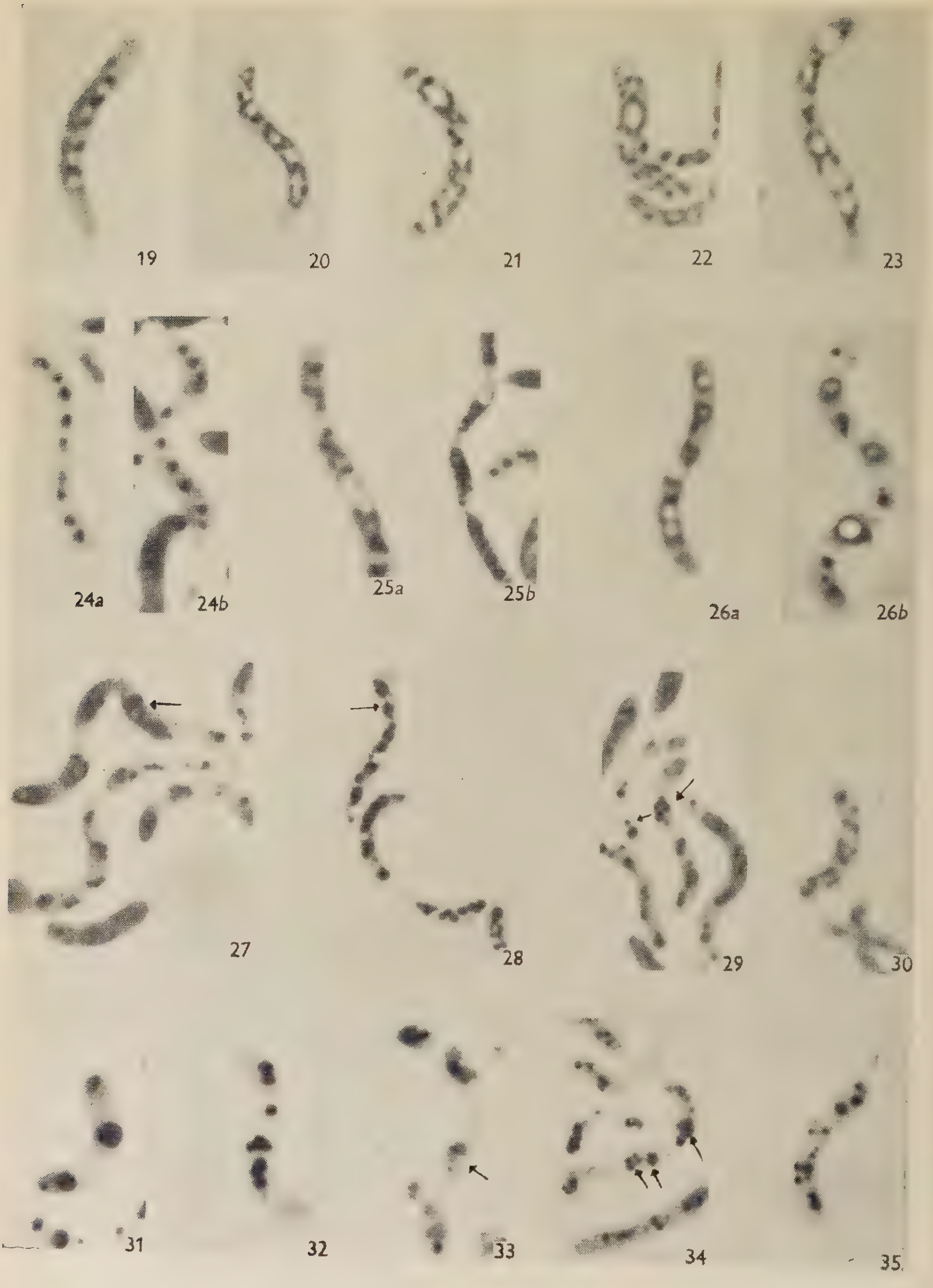
M. A. Williams—Chromatin patterns in *S. anulus*. Plate 1

(*Facing p.* 120)



19 20 21 22 23

24a 24b 25a 25b 26a 26b

27 28 29 30

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M. A. Williams—Chromatin patterns in *S. anulus*. Plate 2

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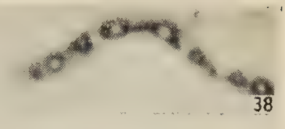
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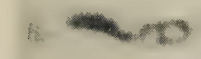
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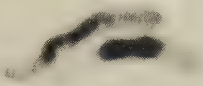
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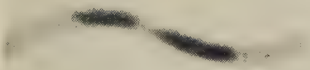
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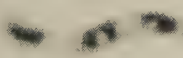
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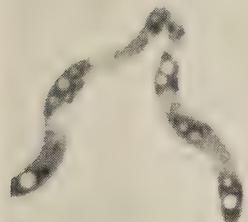
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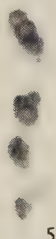
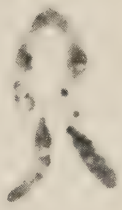
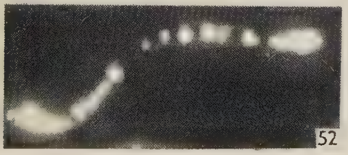

50*a*

50*b*


51*a*

51*b*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M. A. WILLIAMS—CHROMATIN PATTERNS IN *S. ANULUS*. PLATE 3

ROBINOW, C. F. (1944). Cytological observations on *Bact. coli, Proteus vulgaris* and various aerobic spore-forming bacteria with special reference to the nuclear structure. *J. Hyg., Camb.* **43**, 413.

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EXPLANATION OF PLATES

All photographs are of *Spirillum anulus*

PLATE 1

Figs. 1–3. Acid-Giemsa cells from 10, 4 and 8 hr. cultures, respectively, showing undifferentiated appearance of cells despite 15 min. of acid hydrolysis. × 3200.

Figs. 4, 5. Phase-contrast photographs of living cells from 10 and 12 hr. cultures, respectively, showing undifferentiated appearance of early cells. × 3200.

Figs. 6, 7. Acid-Giemsa cells from 48 and 30 hr. cultures, respectively, showing cell division and the ½ and 1½ curved forms of the division cells. × 3600.

Figs. 8, 9. Phase-contrast photographs of living cells from 16 and 14 hr. cultures, respectively, showing cell division and the ½ and 1½ curved forms of the dividing cells. × 3600.

Figs. 10–14 are all of acid-Giemsa cells

Fig. 10. Cell from 12 hr. culture showing axial filament of chromatin material and dark staining of the cytoplasm. × 4000.

Fig. 11. Cell from 14 hr. culture showing fragmentation of the axial filament into thick bars. × 4000.

Fig. 12. Cell from 16 hr. culture showing spherical forms of the chromatin material. × 4000.

Fig. 13. Cell from 16 hr. culture showing curved bar-like forms originating from spherical bodies. × 4000.

Fig. 14. Cells from 16 hr. culture showing division of spherical bodies as well as cell division. × 4000.

Figs. 15–18 are all of living cells

Fig. 15. Phase-contrast photograph of cells from 14 hr. culture showing appearance of the inclusions in the cells; the cytoplasm remains dark. × 3600.

Fig. 16*a*, *b*, Phase-contrast and darkfield photographs of the same cell from a 16 hr. culture showing the appearance of the inclusions as empty vacuoles in the phase-contrast photograph (16*a*) and their appearance as solid spherical bodies by darkfield. Notice that it is not possible to differentiate the periphery of the inclusions from the dark cytoplasm in the phase contrast photograph. × 3600.

Figs. 17, 18. Phase-contrast photographs of cells from an 18 hr. culture showing the inclusions and the lighter cytoplasm of the cells at this time interval. The inclusions appear as dark spheres and as rings of dark material enclosing a lighter material. × 3600.

PLATE 2

Figs. 19–23 are of acid-Giemsa cells

Fig. 19. Cell from 18 hr. culture showing horizontal bar-like chromatin material. × 4000.

Fig. 20. Cell from 20 hr. culture showing separation of horizontal bars into V-forms. × 4000.

Fig. 21. Cell from 22 hr. culture showing chromatin material as a broken ring, which may indicate method of division of this chromatin configuration. Intermediate X-, Y-, and V-forms are seen in the lower part of cell. × 4000.

Fig. 22. Cell from 24 hr. culture showing two rings of chromatin material, surrounding an achromatic core. Small interconnected bead-like granules are seen in the lower part of cell. × 4000.

Fig. 23. Cell from 26 hr. culture showing several ring-like forms, surrounding an achromatic core. Possible division forms are seen in several of these chromatin structures. × 4000.

Figs. 24*a*, *b*. Acid Giemsa and living cell photographed by phase contrast, respectively, showing an exact correlation between the fixed and living images of the chromatin bodies. From an 18 hr. culture. × 3600.

Figs. 25*a*, *b*. Acid-Giemsa and living cell photographed by phase contrast, from an 18 hr. culture, showing the correlation between the horizontal bar-like forms. × 3600.

Fig. 26*a*. Acid-Giemsa cell from 26 hr. culture showing ring forms. × 3600.

Fig. 26*b*. Living cell photographed by phase contrast showing apparent correlation between the same forms of ring-like bodies in living cells. (See text for a discussion of the visual appearance of the large ring-like inclusions.) Small inclusions show apparent budding. × 3600.

Fig. 27. Living cells, photographed by phase contrast, from a 24 hr. culture showing ring-like forms in upper cell (marked with an arrow) and small bead-like granules in lower cells. × 3600.

Fig. 28. Fixed, Giemsa-stained but unhydrolysed cells from same 24 hr. culture as cells in fig. 27, showing spherical form of large ring-like inclusions (upper cell, marked with an arrow) and the correlation between the small bead-like granules of the living cell. × 3600.

Fig. 29. Phase-contrast photograph of cells from 24 hr. culture showing ring-like form of bead-like granules (marked with an arrow in 2nd cell from right) as well as budding (marked with an arrow in 2nd cell from left). × 3600.

Fig. 30. Acid-Giemsa cell from 24 hr. culture showing ring formation of bead-like granules as well as discrete granules. × 3600.

Figs. 31–35. Phase-contrast photographs of living cells

Fig. 31. Cell from a 20 hr. culture showing large spherical inclusions. × 3600.

Fig. 32. Cell from 22 hr. culture showing division of large spherical inclusions (upper inclusion); the subdivision of the small inclusions into more than one body (middle inclusions) and the formation of the semicircular bodies from the inclusions. × 3600.

Fig. 33. Cell from 22 hr. culture showing semicircular forms with budding of small bead-like granules from inclusions marked with an arrow. × 3600.

Fig. 34. Cells from 24 hr. culture showing formation of rings of bead-like granules from spherical inclusions (marked with arrows). × 3200.

Fig. 35. Cell from 26 hr. culture showing spherical inclusions (upper part of cell) and formation of V-forms from these bodies. × 3600.

PLATE 3

Figs. 36–48 are of acid-Giemsa cells

Figs. 36. Cells from 30 hr. culture showing condensation of bead-like granules into bars. Cell division is occurring at the centre. × 4000.

Fig. 37. Cell from 32 hr. culture showing aggregation of chromatin material at edges of vacuoles. × 4000.

Fig. 38. Cell from 34 hr. culture showing a series of connected rings of chromatin material. × 4000.

Fig. 39. Cell from 38 hr. culture, showing aggregation of chromatin material into an axial filament, around the vacuoles of the cell. × 4000.

Figs. 40–41. Cells from 40 hr. culture showing compact arrangement of chromatin material at centre of the cells in the form of a solidly staining mass. × 4000.

Fig. 42. Cell from 42 hr. culture showing division of chromatin aggregation. × 4000.

Fig. 43. Cell from 42 hr. culture showing division of chromatin mass coinciding with cell division. × 4000.

Fig. 44. Cell from 42 hr. culture showing extension of chromatin material, along the vacuoles of the cells, of the compact chromatin mass. × 3600.

Fig. 45. Cell from 44 hr. culture showing formation of spherical bodies and curved bars from the axial filament of chromatin material. × 3600.

Fig. 46. Cell from 44 hr. culture showing division of chromatin bars. × 4000.

Fig. 47. Cell from 46 hr. culture showing horizontal bars. × 4000.

Fig. 48. Cell from 48 hr. culture showing rings and chains of bead-like granules. × 4000.

Figs. 49–52. Living cells photographed by phase contrast and darkfield microscopy.

Fig. 49. Cell from 38 hr. culture showing clusters of inclusions as well as difficulty in differentiating the individual inclusions. These clusters may give rise to the compact chromatin aggregations of the acid-Giemsa cells. × 3600.

Figs. 50*a*, *b*. Phase-contrast and darkfield photographs of the same cells from a 36 hr. culture, showing clusters of inclusions; the difficulty of differentiating the inclusions in the phase-contrast photograph (50*a*) which appear to merge. The discreteness of the inclusions is shown in the darkfield photograph (50*b*). × 3600.

Fig. 51*a*, *b*. Phase-contrast and darkfield photographs of the same cells from a 30 hr. culture showing arrangement of inclusions which might give rise to the axial filaments in the acid-Giemsa cells. Merging of the inclusions in lower cells is shown in phase-contrast photograph (51*a*) but darkfield photograph (51*b*) shows these bodies to be discrete. × 3600.

Fig. 52. Darkfield photograph of cell from 32 hr. culture showing close spacing of inclusions which might give rise to the appearance of an axial filament in the acid-Giemsa cells. × 3600.

Fig. 53. Phase-contrast photograph of cell from 30 hr. culture showing discrete arrangement of inclusions and obvious photographic differences in the type of inclusions. The two spherical dark inclusions, at either side of the brightly refractile inclusion at right of cell, appear to merge with this body. × 3600.

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The Localization of the Cell-Bound Penicillinase of *Bacillus cereus* in Protoplasts

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SUMMARY: Protoplasts of *Bacillus cereus* 569 and *B. cereus* 569/H were shown to be as competent as intact cells in the induced and constitutive formation of penicillinase, as well as in other biochemical activities. It was demonstrated that the cell-bound penicillinase was localized at or near the cell surface. The β-penicillinase fraction was released by digestion of the cell wall and by washing of the protoplast membrane. However, the γ-penicillinase was tightly bound to the cytoplasmic membrane. It was obtained in a soluble form by treating the membrane with sonic vibration or with fat solvents.

It was demonstrated by Pollock (1956) that of the total penicillinase formed constitutively by *Bacillus cereus* 569/H, and as a result of induction by *B. cereus* 569, about 85% was exocellular (α-penicillinase). β-penicillinase, the fraction of cell-bound enzyme which could be neutralized by anti-exo-penicillinase serum, could be partially removed from the cell by washing; but the γ-penicillinase, which is an immunologically and physicochemically distinct fraction, was very tightly associated with the cell. Attempts to obtain the γ-penicillinase in a soluble form were only partially successful. The observation that such soluble enzyme was obtained only after disruption of the intact organisms suggested that some of the γ-penicillinase might exist as a soluble cytoplasmic constituent. However, the results did not permit the exclusion of the possibility that all the enzyme was bound at the surface of the cell and was released when the surface structure was disintegrated.

In the course of an investigation of the formation of penicillinase in protoplasts of *Bacillus cereus* it was demonstrated that all cell-bound enzyme was located at the cell surface. A portion, β-penicillinase, was associated with the cell wall and could be removed by dissolution of the wall; the γ-penicillinase was exclusively localized in or at the cell membrane. Little or no penicillinase could be detected in the cell cytoplasm.

METHODS

Organisms. Bacillus cereus, NRRL 569, which can be induced to form penicillinase (Pollock, 1950). *B. cereus* 569/H, a mutant strain derived from NRRL 569, which produces this enzyme constitutively (Kogut, Pollock & Tridgell, 1956).

Media and conditions of growth. Organisms were grown in the casein hydrolysate medium described by Pollock (1957). In some instances sodium citrate was omitted without effect on the growth of the organism or on the other properties under investigation. Medium was automatically inoculated with a suspension of spores (Pollock, 1953) which had been allowed to germinate in stationary culture at 35° for 10–12 hr. The inoculated culture was then shaken at 35°.

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The automatic inoculation was achieved using the apparatus designed by Dr H. J. Rogers and Dr D. A. Lowther. The spore suspension was placed in a 25 ml. separatory funnel which was connected to a receiving flask by rubber tubing. The rubber tubing was held closed by two metal strips which were firmly screwed together. One of these strips was connected by an armature to a solenoid, which in turn was connected to an automatic interval timer. To permit free flow through the rubber tubing the metal strip was pulled back by the magnetic force of the solenoid, which was energized by the preset timer.

Preparation of protoplasts from Bacillus cereus. Protoplasts of *B. cereus* 569 and *B. cereus* 569/H were prepared by a method based on that described by Dark & Strange (1957). Modifications were necessitated by the fact that these organisms were prone to lysis when subjected to repeated washing in dilute buffer solutions, and the protoplasts were unstable in the absence of magnesium ion. The final procedure adopted was as follows.

Organisms grown to the exponential phase (stationary phase organisms were relatively insensitive to protoplast formation by the method used) were harvested by centrifugation; washed once with a solution of McIlvaine's buffer (pH 7·0; Na_2HPO_4, 0·017 M and citric acid, 0·018 M, Clark, 1928) containing glucose (1 %, w/v) + $MgSO_4$, 0·05 M and resuspended in a solution of the following: sucrose, 0·3 M; $CoSO_4$, $8{\cdot}5 \times 10^{-4}$ M; $MgSO_4$, 0·05 M; sodium citrate, 5×10^{-3} M; potassium phosphate buffer (pH 7·0), 0·01 M; glucose, 1 % (w/v); 2 ml. of a solution of lytic enzyme (see below) in 5 ml. of reaction mixture. The organisms were incubated without shaking at 37°.

When the formation of protoplasts was complete, as judged by their appearance by phase-contrast microscopy, they were collected by centrifugation and resuspended as indicated in the text. To assess the degree of protoplast formation a portion of the suspension was diluted ten times with water, thereby lysing the protoplasts and leaving intact the unaltered organisms. The optical density of this diluted suspension was compared with that of a similar suspension of a control culture of organisms incubated in the absence of lytic enzyme. The difference between the optical densities of these two suspensions was taken as a measure of the proportion of the organisms converted to protoplasts.

These microscopic and optical density methods for measuring protoplast formation would not necessarily indicate the presence of very small numbers of residual cells. In order to be certain that the biochemical activities of a protoplast preparation was not due to such intact cells, a cell control was included in all experiments designed to study the synthetic and metabolic properties of protoplasts and protoplast substructures, i.e. the activity of a protoplast suspension was compared with that of a protoplast suspension lysed with water, and therefore containing only intact cells. Complete

(99–100 %) conversion of cells to protoplasts was concluded to have occurred if (*a*) no intact cells could be detected with phase-contrast microscopy, (*b*) the optical density of a lysed protoplast preparation approached zero, (*c*) the penicillinase-forming ability of a water-lysed preparation was zero.

Preparation of lytic enzymes for protoplast formation. Two lytic enzymes, which attack the cell wall of *Bacillus cereus* 569 and *B. cereus* 569/H, were isolated from the former organism, essentially by the procedure described by Strange & Dark (1957*b*). Complete sporulation and the onset of spore release (judged by phase-contrast microscopy) was attained within 19–24 hr. when cultures of *B. cereus* 569 in 'S'-broth (Pollock, 1953) were shaken at 35° in a conical flask, the volume of which was ten times that of the culture volume. The cultures were shaken on a reciprocating shaker described by Kantorowicz (1951), at a speed of 85 strokes/min. on a rack moving with an amplitude of 3 in./stroke. Under these conditions optimum aeration was ensured. Washed organisms were incubated without shaking at pH 5·6 with toluene at 37° for 2 hr. The filtered supernatant fluid was active in forming protoplasts. However, the dissolved toluene produced lysis of the protoplasts; thus partial isolation of the enzymes was necessary. The spore coat enzyme (S enzyme) was isolated by precipitation at pH 3 using McIlvaine's buffer (composed of Na_2HPO_4, 0·004 M + citric acid, 0·08 M; Clark, 1928). The precipitate was dissolved in a solution of sodium bicarbonate (0·01 M) + sodium citrate (0·05 M). The enzyme was reprecipitated at pH 3, redissolved in a minimum volume of the bicarbonate + citrate solution and stored at −20°. The vegetative cell enzyme (V enzyme) was obtained from the supernatant fluid of the first pH 3 precipitation by saturating it with solid ammonium sulphate and leaving for several hr. at 2°. The precipitate formed was collected by filtration. It was dissolved in bicarbonate + citrate solution and was dialysed overnight at 2° against distilled water. The dialysed solution was stored at −20°. When stored in this way the S and V enzymes were stable for at least 5 months.

Penicillinase assay. Total penicillinase activity was measured by the method of Henry & Housewright (1947) in the presence of 0·3 % gelatin. It was established that the various components of the media and buffer mixtures used had no effect on the assay. To measure the amount of extracellular α-penicillinase and cell-bound β-penicillinase present in any sample, 1 ml. portions were pre-incubated at room temperature in Warburg flasks with 0·5 ml. of a solution of anti-α-penicillinase serum (Pollock, 1956). The antibody was added in a concentration three times that required for maximal neutralization of enzyme present. The final volume of assay mixture was adjusted to 3·0 ml.

γ-Penicillinase. The non-neutralizable enzyme activity was taken as a measure of the amount of γ-penicillinase present, after allowing for the residual activity of the enzyme-antibody complex. It was established that the penicillinase activity not neutralized by anti-exoenzyme serum was rapidly inactivated by iodine (Pollock, 1956), by using the iodometric assay system described by Citri (1958).

RESULTS

Protoplasts of Bacillus cereus

When the S enzyme or the V enzyme (isolated from *Bacillus cereus* 569), both of which attack cell wall material, was allowed to act in a medium of high osmotic pressure on either *B. cereus* 569 or *B. cereus* 569/H, the structural integrity of the organisms disappeared. It was seen by phase-contrast microscopy that the bacillary form of the organism was lost and was replaced by a spherical form which was highly sensitive to osmotic shock.

Of the several chemical tests for protoplasts put forward by Brenner *et al.* (1958) none has yet been applied. However, electron microscopy of the spherical forms obtained (Pl. 1, fig. 1) indicated that these structures are devoid of adhering fragments of cell wall. Remnants of cell wall when present were clearly seen as, for example, in the case of the spore seen in Pl. 1, fig. 2, still encased by cell wall. The electron micrographs of the spherical forms obtained from *Bacillus cereus* 569 and *B. cereus* 569/H are similar to those of the denuded protoplasts derived from *Bacillus megaterium* by the action of lysozyme (McQuillen, 1959). They are quite unlike the micrographs of the spherical forms obtained when *Escherichia coli* is grown in the presence of penicillin, which were shown by McQuillen (1958) to be enveloped in fragments of cell wall. These microscopical observations, together with the finding of Strange & Dark (1957*a*, *b*) that the lytic enzymes derived from sporulated *Bacillus cereus* produce almost complete digestion of vegetative cell walls, make it likely that naked protoplasts are indeed obtained by the procedure used in the present investigation.

Biochemical capabilities of protoplasts of Bacillus cereus

Studies of the biochemical activities of protoplasts of *Bacillus cereus* 569 and *B. cereus* 569/H confirmed findings obtained with protoplasts of other micro-organisms (McQuillen, 1958). These subcellular structures oxidize endogenous substrates and added glucose and glutamate at approximately the same rate as do intact organisms, incorporate amino acids into their proteins and utilize glycine for the formation of nucleic acid purines. When the protoplasts of *B. cereus* were incubated in a growth medium supplemented with sucrose (0·3 M) and magnesium ions (0·05 M), their volume increased several fold, but no division of protoplasts or reversion to the bacillary form was observed during 3½ hr. Constitutive penicillinase formation was demonstrated in protoplasts of *B. cereus* 569/H. The observation of Kramer & Straub (1955) that preinduced *B. cereus* 569 when converted to the spherical forms (by the use of lysozyme) formed enzyme, was confirmed. It was also found that protoplasts could be directly induced to form penicillinase. In the case of both constitutive and induced enzyme synthesis, the rate and extent of enzyme formation by protoplasts was of the same order as that exhibited by intact organisms. The release of exocellular α-penicillinase to the medium was unimpaired by removal of the cell wall; for of the total penicillinase synthesized by protoplasts of *B. cereus* 569 and 569/H, and by intact organisms, the same proportion was formed as exo-enzyme in each case.

The localization of penicillinase in protoplasts

Weibull (1956) showed that lysis of protoplasts in a hypotonic medium containing magnesium ions, gave rise to intact cell membranes. This finding made possible a limited step-wise dissection of the bacterium. This technique has been applied in a study of the distribution of penicillinase among the cell fractions of *Bacillus cereus*.

The procedure employed and the fractions obtained are described in Fig. 1. Protoplasts of *Bacillus cereus* 569 and *B. cereus* 569/H, when resuspended in distilled water, were completely disrupted. Electron microscopy revealed that the resulting suspension contained large particles which could be sedimented at 15,000 *g*, smaller particles which sedimented at 30,000 *g* and particles which were brought down at 105,000 *g*. The final supernatant fluid was particle-free.

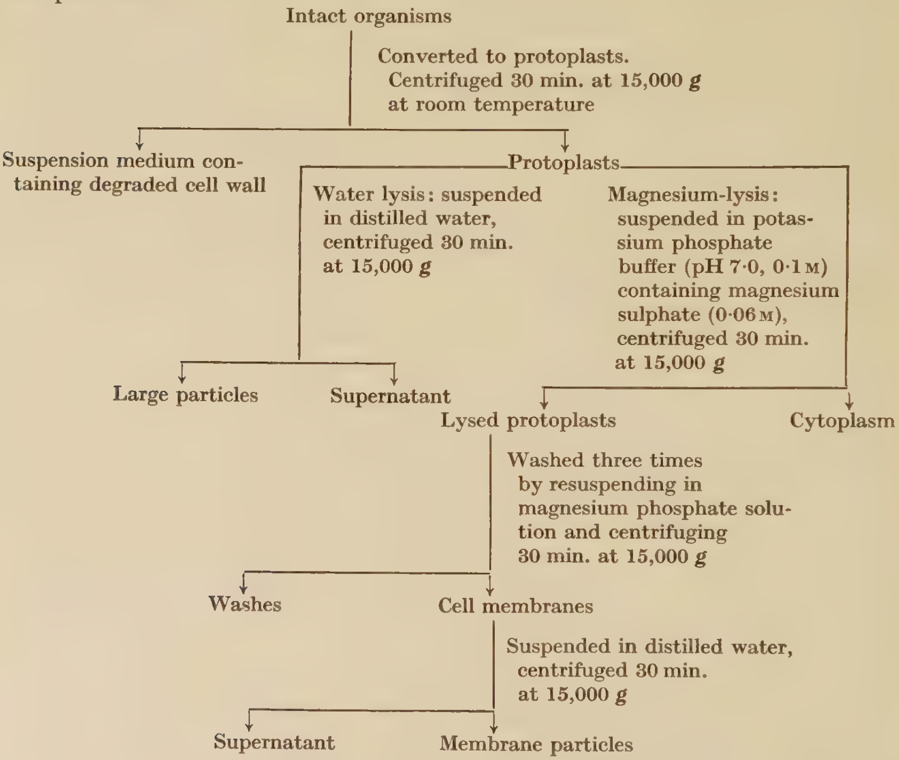
Intact organisms
Converted to protoplasts. Centrifuged 30 min. at 15,000 g at room temperature
Suspension medium containing degraded cell wall
Protoplasts
Water lysis: suspended in distilled water, centrifuged 30 min. at 15,000 g
Magnesium-lysis: suspended in potassium phosphate buffer (pH 7·0, 0·1 M) containing magnesium sulphate (0·06 M), centrifuged 30 min. at 15,000 g
Large particles
Supernatant
Lysed protoplasts
Cytoplasm
Washed three times by resuspending in magnesium phosphate solution and centrifuging 30 min. at 15,000 g
Washes
Cell membranes
Suspended in distilled water, centrifuged 30 min. at 15,000 g
Supernatant
Membrane particles

Fig. 1. Procedure for the isolation of cell fractions from *Bacillus cereus*. All manipulations for the fractionation of protoplasts were carried out at 2°.

However, when the protoplasts were suspended in a solution of magnesium ions ($MgSO_4$, 0·05 M in potassium phosphate buffer pH 7·0, 0·01 M), there occurred lysis of a type such that much of the intracellular protein and RNA was released into the medium, although the cytoplasmic membrane retained its structural integrity. These membranes were harvested by centrifugation at 15,000 *g*. A preparation of thrice-washed membranes is seen in Pl. 1, fig. 3. When suspended in water the membrane structure disintegrated, giving rise to a considerable quantity of particles which sedimented at 15,000 *g* and 30,000 *g*, but little or no material which sedimented at 105,000 *g*.

In Fig. 2 are presented the results of a study of the distribution of penicillinase activity in cell fractions of *Bacillus cereus* 569/H. Similar results were obtained using induced *B. cereus* 569. It is clear that of the penicillinase associated with once-washed organisms (i.e. the cell-bound enzyme described by Pollock, 1956), half was released as a result of the digestion of the cell wall and half remained bound to the resulting protoplasts. Over 90 % of the protoplast-bound enzyme was recovered in the cell membranes, and of this almost all was found in the large particles which were formed when the membranes were disintegrated. Taking into account the fragility of the membrane and the inaccuracies inherent in the methods of separation of the cell fractions, it seems most probable that the protoplast-bound penicillinase of *B. cereus* 569 and 569/H is localized in or on the cell membrane. Little can be detected in the cytoplasm.

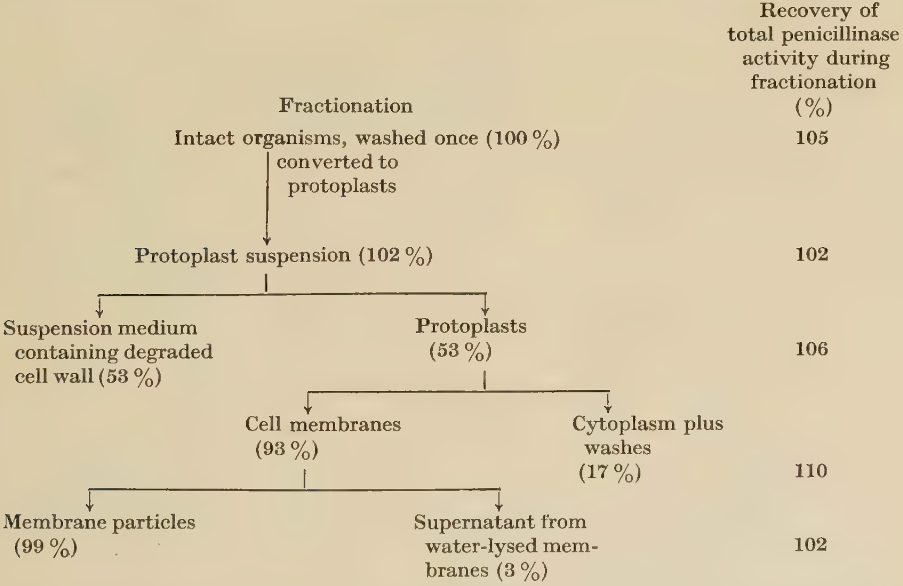
Recovery of total penicillinase activity during fractionation (%)
Fractionation
Intact organisms, washed once (100 %)
105
converted to protoplasts
Protoplast suspension (102 %)
102
Suspension medium containing degraded cell wall (53 %)
Protoplasts (53 %)
106
Cell membranes (93 %)
Cytoplasm plus washes (17 %)
110
Membrane particles (99 %)
Supernatant from water-lysed membranes (3 %)
102

Fig. 2. Distribution of penicillinase activity in fractions of *Bacillus cereus* 569/H. Fractions were obtained as described in Fig. 1. The figures in parentheses indicate that percentage of the penicillinase activity of the previous fraction recovered in the fraction noted before the bracket.

Nature of the penicillinase in cell fractions of Bacillus cereus

Pollock (1956) showed that the penicillinase activity of intact *Bacillus cereus* is due to the presence of two distinct protein entities: (*a*) β-penicillinase which is detected by its inactivation by antiserum to the α-penicillinase, and by its relative iodine insensitivity; (*b*) γ-penicillinase which is unaffected by this antiserum, but is inactivated by iodine. A study of the sensitivity of the penicillinase in cell fractions of *B. cereus* 569/H to anti-α-serum (Table 1) revealed that whereas the penicillinase activity of intact organisms, protoplasts and unwashed membranes was partially neutralized (*c.* 15 %) by the antiserum, that of the washed membranes and particles derived from them was totally unaffected by the serum. It was further demonstrated (Table 1) that the non-neutralizable penicillinase of the membrane preparations was very rapidly inactivated by iodine, since in the iodometric assay test (Citri, 1958) no decolorization of iodine by these preparations was observed even after 24 hr. Under the conditions of the assay, an amount of α- or β-penicillinase of equivalent activity decolorized the iodine within 5–10 min. It would seem, therefore, that the penicillinase of the membranes is γ-penicillinase, and that in the intact organisms and protoplasts a small amount of β-penicillinase is adsorbed in a form which can readily be removed by washing.

Table 1. *Sensitivity of the penicillinase activity of cell fractions of* Bacillus cereus 569/*H to anti-exopenicillinase serum and to iodine*

Expt. no.	Fraction	Percentage neutralization by anti-α-serum (experimental observation)	Percentage unneutralizable penicillinase activity*	Decolorization of iodine
1	Intact organisms, washed once	16	78	Complete within few minutes
2	Protoplasts, unwashed	11	81	Complete within few minutes
	Membranes, unwashed	16	78	Complete within few minutes
	Large particles obtained by water-lysis of unwashed membranes	0	100	Not tested
	Supernatant from water-lysed unwashed membranes	19	88	Not tested
3	Membranes, washed 3 times	0	100	None, after 24 hr.
	Wash from membranes	14	75	Not tested
	Large particles from membranes	0	100	None, after 24 hr.
	Supernatant from water-lysed membranes	0	100	None, after 24 hr.

* The percentage unneutralizable penicillinase activity was calculated by subtracting the neutralizable activity, corrected for the residual activity of the antibody-enzyme complex, from the total.

Attempts to obtain soluble γ-penicillinase

By disruption of cellular integrity (by autolysis or crushing in the Hughes' press) Pollock (1956) obtained a portion of the γ-penicillinase (*c.* 25–50 %) of *Bacillus cereus* 569 in a soluble state (i.e. non-sedimentable at 105,000 *g*). In the present study about 5 % of γ-penicillinase produced by lysing protoplasts was soluble. However, by treatment of isolated cell membranes of *B. cereus* 569/H with sonic vibrations it was possible to obtain all of the γ-penicillinase in a soluble form (Fig. 3). It was found that when washed membrane particles were subjected to sonic vibrations in the presence of 0·1 % gelatin at 2°, about 30 % of the total penicillinase activity of the preparation was lost after 1 hr. However, of the activity remaining, a portion was detectable in the supernatant fluid obtained after centrifugation at 105,000 *g* within 5 min. after treatment was begun, and after 30 min. almost all the activity was recovered in this supernatant fluid. The insensitivity of both membrane and soluble enzyme activity to anti-α-penicillinase serum, and the sensitivity to iodine, indicated that both the particulate and soluble enzyme under study was γ-penicillinase. It is clear that the cell-bound γ-penicillinase was removed from the membrane as soluble γ-penicillinase, by sonic vibrations. Studies with butanol and toluene indicated that these fat solvents were also active in releasing γ-penicillinase from the membrane in a soluble and active state.

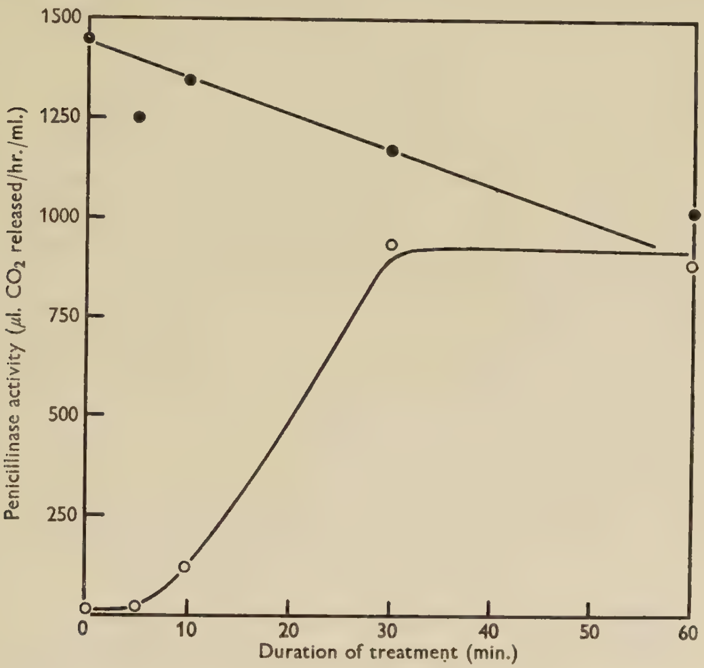

Fig. 3. Release of γ-penicillinase from washed membranes of *Bacillus cereus* 569/H in a soluble form, by treatment with sonic vibrations. A membrane preparation in 0·1 % gelatin (initial volume 11 ml.), was treated with sonic vibrations (using a 25 kc. oscillator) at 2°. At the noted times 2 ml. portions were removed and chilled. To each sample 12 ml. of 0·2 % gelatin was added. The penicillinase activity of 1 ml. portions was measured and the remaining 13 ml. was centrifuged at 2° for 1 hr. at 105,000 *g*. The enzyme activity of the supernatant and the pellet (resuspended in 0·2 % gelatin) was determined. ●—●, activity of whole preparation; ○—○, activity of supernatants.

DISCUSSION

The demonstration by Weibull (1953) that treatment of *Bacillus megaterium* KM in hypertonic medium with lysozyme resulted in the degradation of the cell wall and the release of protoplasts marked a new phase in the study of cell physiology and biochemistry. Such controlled morphological dissection of bacteria made feasible an extension of the investigation of the structural basis of cellular activity, previously carried out by the techniques of light and electron microscopy, histochemistry, differential centrifugation of cellular components, etc. (see Sjöstrand, 1957; Gomori, 1957; Hogeboom, 1955). In particular it became possible to isolate the cell membrane and to analyse directly its chemical, biochemical and physiological properties. The chemical evidence accumulated to date (Weibull, 1957; Mitchell & Moyle, 1956*a*, *b*; Gilby, Few & McQuillen, 1958; Gilby & Few, 1958) indicates that, for three different species of bacterium, the cytoplasmic membrane is lipoprotein in nature and contains small amounts of carbohydrate material.

Of very great interest is the finding that the cell membrane exhibits a large number of enzyme activities. Weibull (1953) and Mitchell & Moyle (1956*a*) showed that the cytochrome system of *Bacillus megaterium* and *Staphylococcus aureus*, respectively, are localized in the protoplast membrane, or particles derived therefrom. The latter authors showed that the major portion of the acid phosphatase and the succinic and lactic acid dehydrogenases of *S. aureus* are to be found in particles thought to come from the cell membrane. Storck & Wachsman (1957*a*, *b*) located all of the succinic, lactic and α-ketoglutaric dehydrogenase activities and half of the malic dehydrogenase activity in the cytoplasmic membrane of *B. megaterium*.

In the present study it has been found that essentially all of the cell-bound penicillinase of *Bacillus cereus* 569 and *B. cereus* 569/H is associated with the cell membrane and cell wall. The β-penicillinase can be completely removed by extensive washing. This suggests that this fraction is loosely adsorbed at the cell surface. The γ-penicillinase on the other hand is very firmly attached to the cell membrane, and is only released in a soluble form as a result of disruption of the membrane, e.g. by mechanical disintegration or by treatment with lipid solvents. The observation that butanol and toluene are effective in dissolving the γ-penicillinase away from the membrane suggests that this enzyme may be present in the membrane in lipo-protein binding.

The detection of the various enzymes discussed above, in the cytoplasmic membrane, raises the question of the site of their synthesis. The demonstration that the penicillinase of *Bacillus cereus* is present in the extracellular medium, or else is to be found closely associated with the cytoplasmic membrane and cell wall, and is barely, if at all, detectable in the cytoplasm, does not exclude the possibility that these enzymes are formed within the cell and are later transported into and across the membrane. However, it seems rather more likely that the cytoplasmic membrane itself may be the site of such enzyme formation. Indeed the experiments of Spiegelman (1957), Hunter, Crathorn & Butler (1957), Nisman, Bergmann & Berg (1957), Butler, Crathorn & Hunter (1958) and Brown & Brown (1958) suggest that the cell membrane may be directly involved in the early stages of protein synthesis (i.e. activation of amino acids and transfer to an acceptor site), and that it may also participate in fashioning the final protein. Preliminary experiments have shown that penicillinase formation occurs in partially-lysed protoplasts of *B. cereus*. These studies are to be extended in an effort to throw some light on these problems.

The author wishes to express her thanks to all the members of the Division of Bacterial Physiology, and in particular to Dr M. R. Pollock and Dr H. J. Rogers, for their kind hospitality, their continued help and critical discussion throughout her stay. The technical assistance of Mr N. Dickens is gratefully acknowledged. The author is a fellow of the National Cancer Institute of Canada.

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EXPLANATION OF PLATE

Figs. 1, 2. Electron micrographs of preparations of *Bacillus cereus* 569/H. Fig. 1. Protoplasts, prepared as described under Methods. Fig. 2. Protoplast to left of spore. Magnification, *c.* × 16,000. The electron micrographs were taken by Dr R. C. Valentine.

Fig. 3. Electron micrograph (taken by Dr R. C. Valentine) of a protoplast membrane of *Bacillus cereus* 569/H. Magnification, *c.* 16,000.

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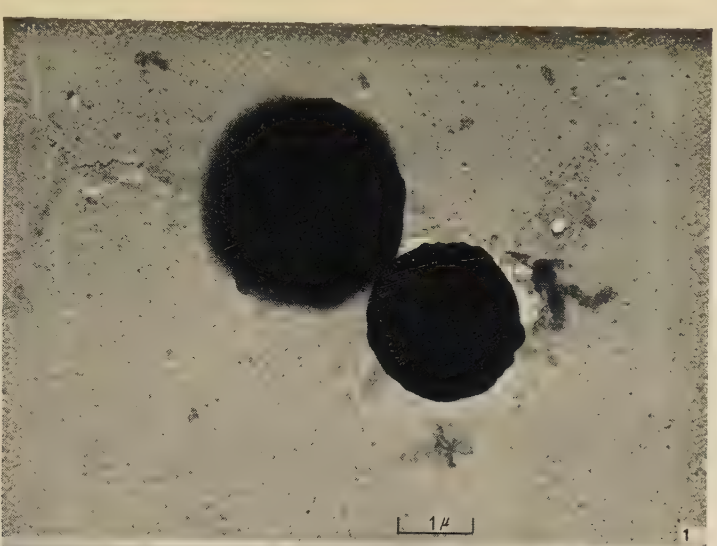

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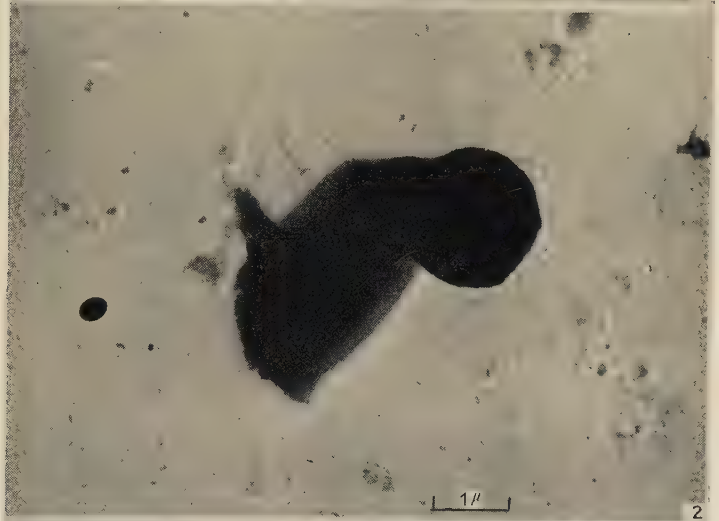

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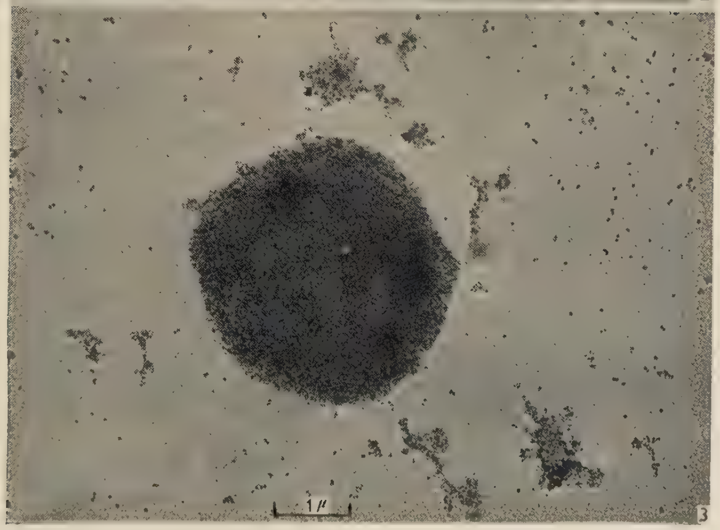

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R. Sheinin—Penicillinase in protoplasts

(*Facing p.* 134)

Hertman, I. & Ben-Gurion, R. (1958). *J. gen. Microbiol.* **21**, 135–143

A Study on Pesticin Biosynthesis

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SUMMARY: Strains of *Pasteurella pestis* under certain conditions form a bacteriocin-like material, which has been named pesticin. Pesticin formation was induced by ultraviolet irradiation. A chemically defined medium composed of inorganic salts and amino acids supported the synthesis of pesticin by irradiated organisms. The minimal amino acid requirement for pesticin synthesis has been established. For the synthesis of pesticin at 37° the irradiated organisms required certain amino acids which they did not require at 27°. Bacteria in the stationary phase and actively dividing bacteria were equally able to produce pesticin upon irradiation. Concentrations of chloramphenicol lower than those required for bacteriostasis inhibited pesticin synthesis. Non-pesticin producing mutants of *P. pestis* were obtained by exposure of the bacteria to higher doses of u.v. irradiation than those required for the induction of pesticin formation. These mutants were sensitive to pesticin.

It was reported previously (Ben-Gurion & Hertman, 1958) that ultraviolet (u.v.) irradiated *Pasteurella pestis* produces a bacteriocin-like material which we named pesticin, when incubated in Proteose peptone broth, while no pesticin was detected after incubation in phosphate buffer. In view of the apparent protein nature of pesticin (Ben-Gurion & Hertman, 1958) the way of its production can be considered as a problem in protein synthesis.

Pasteurella pestis exhibits different amino acid requirements for growth in defined media at 27° as compared with 37° (Hills & Spurr, 1952; Higuchi & Carlin, 1958). The synthesis of a number of antigens of *P. pestis* is also known to be temperature dependent (Crumpton & Davies, 1956; Fox & Higuchi, 1958). The present study has been mainly concerned with the replacement of the complex proteose peptone medium, in which pesticin synthesis has been observed, by a chemically defined medium.

METHODS

Organisms. The following strains were used: a streptomycin-resistant mutant of *Pasteurella pestis* Kimberley strain P; and *P. pseudotuberculosis* No. 134 C.

Media. Proteose peptone broth (Pr3); Proteose peptone agar (Pr3A); blood agar base (BAB); phosphate buffer M/15. These media were prepared as described previously (Ben-Gurion & Hertman, 1958).

Casein hydrolysate mineral glucose medium (CHMG) (Englesberg & Levy, 1954) had the following composition (%, w/v): vitamin-free Casamino acids (Difco), 3; glucose, 0·2; NH_4Cl, 0·1; $CaCl_2$, 0·001; $FeCl_3.6H_2O$, 0·00025; $MgSO_4.3H_2O$, 0·035; $KH_2PO_4+Na_2HPO_4$ buffer, pH 7, 0·025 M. The

Casamino acids were dissolved in distilled water. Glucose was sterilized separately by autoclaving and added aseptically.

Amino acid media. The composition of these media was otherwise that of the CHMG medium, but the vitamin-free Casamino acids were replaced by mixtures of known amino acids. Each amino acid was used in a concentration of 0·004 % (w/v). The amino acids were purchased from Schwartz Laboratories Inc., Mt. Vernon, N.Y. The purity of the amino acids was ascertained chromatographically.

The conditions of cultivation, count of colony formers, u.v. irradiation were as described previously (Ben-Gurion & Hertman, 1958).

Pesticin titration. The pesticin was titrated by spotting drops (0·01 ml.) of a series of dilutions of the preparation to be titrated, on the surface of a plate seeded with the indicator strain (*Pasteurella pseudotuberculosis*). The indicator plates were prepared some minutes before the titration by soaking a filter-paper in a suspension of the strain and leaving the paper on the plates for 5 min. The plates were then incubated at 37° for 24 hr. In this way a series of zones of decreasing inhibition was obtained ranging from complete inhibition through partial inhibition to normal growth. The highest dilution that gave a visible inhibition zone was defined as containing one arbitrary unit/ml. (A.U./ml.).

Pesticin biosynthesis. In the general procedure for observing pesticin synthesis *Pasteurella pestis* was grown in Pr3 broth for 18 hr. at 27° until a concentration of 10^7 bacteria/ml. was reached. The growing organisms were washed twice, diluted in phosphate buffer to a concentration of about 10^8 organisms/ml. and u.v. irradiated for 60 sec. Samples of the irradiated bacterial suspension were diluted 1/5 in the amino acid media and incubated for 4 hr. at the desired temperature. Pesticin titrations and bacterial counts were performed during and after the incubation period. In experiments in which both bacterial growth and pesticin synthesis were determined only freshly harvested organisms were used. In experiments where only pesticin synthesis was determined the bacterial suspension could be stored at 5° for 2 days.

RESULTS

Pesticin synthesis in a complete amino acid medium

Preliminary experiments showed that the CHMG medium was as suitable as Pr3 broth for pesticin production by irradiated bacteria. When the vitamin-free Casamino acids were replaced by a mixture of 18 amino acids (L-aspartic acid, L-proline, L-lysine, L-threonine, L-glutamic acid, L-cysteine hydrochloride, L-alanine, L-serine, L-tryptophan, L-histidine, L-methionine, L-valine, L-arginine, L-leucine, L-isoleucine, glycine, L-phenylalanine and L-tyrosine) pesticin synthesis proceeded to the same extent (pesticin yield 750 A.U./ml.) as in the Pr3 broth. No quantitative differences were found in pesticin synthesis at 27° and 37°. This medium was, therefore, called the complete amino acid medium (CAA).

To determine which amino acids are essential for pesticin synthesis, 18 media were made in each of which one of the above amino acids was omitted from the CAA medium. Irradiated bacteria were transferred to these media as well as to the CAA medium, and pesticin synthesis at 27° and 37° measured. The results are presented in Table 1. The omission of methionine, phenylalanine, valine or glycine inhibited pesticin synthesis. Leucine and isoleucine were required at 37° but not at 27°.

Table 1. *Pesticin biosynthesis in CAA medium from which one of the 18 amino acids was omitted*

Samples of 10^8 *Pasteurella pestis* organisms/ml. were diluted 1/5 in 19 amino acid media. Pesticin synthesis after incubation at 27° and 37° for 4 hr. was measured by pesticin titration.

The amino acid omitted from CAA medium	Incubation temperature 27°	37°
	Pesticin concn. (arbitrary units/ml.)	
None (CAA medium)	500	500
L-Methionine	2	2
Glycine	10	10
L-Phenylalanine	10	10
L-Valine	10	2
L-Lysine	500	500
L-Leucine	500	10
L-Isoleucine	500	2

Omission of L-aspartic acid, L-proline, L-glutamic acid, L-cysteine (HCl), L-serine, L-histidine, L-threonine, L-tyrosine, L-arginine, L-alanine and L-tryptophan, did not decrease the pesticin yield at either temperature.

Minimal amino acid medium for pesticin synthesis

To determine the minimal amino acid requirement for pesticin synthesis a medium was developed containing the six amino acids found above to be essential (methionine, valine, phenylalanine, glycine, leucine and isoleucine) This medium did not support pesticin synthesis, but on addition of lysine and arginine synthesis did take place. The eight amino acids methionine, phenylalanine, glycine, valine, leucine, isoleucine, lysine and arginine together were thus found to be essential for pesticin synthesis. The medium containing these amino acids was called the minimal amino acids medium (MAA). Arginine could be omitted at 27° without decreasing the yield of pesticin. Cysteine added to MAA medium doubled the pesticin production. Addition of alanine increased the pesticin yield in MAA medium at 27°. The yield of pesticin in MAA medium was 10% of the yield in the CAA medium containing the 18 amino acids which are found in casein hydrolysate. Quantitative differences were found between pesticin production at 27° and 37°. The yield was always considerably lower at 37° than at 27°. The omission of phenylalanine, lysine, glycine, valine, leucine, isoleucine or methionine from the cysteine-supplemented MAA medium inhibited pesticin synthesis at either temperature. The results of these experiments are presented graphically in Fig. 1. When phenylalanine, valine and methionine were replaced by their D-isomers in MAA medium, no pesticin synthesis was observed.

The role of glucose in pesticin synthesis

Experiments were performed to establish whether the addition of glucose was essential for pesticin synthesis. Glucose-depleted bacteria cells were prepared by growing *Pasteurella pestis* for 48 hr. at 27° in glucose-free casein hydrolysate medium (CHM). These organisms were seeded again into CHM medium and incubated for 18 hr. at 27°. The actively growing organisms were harvested, washed, and pesticin synthesis in CAA medium and in cysteine-supplemented MAA medium was measured. Pesticin synthesis proceeded in the absence of glucose to the same extent as in the glucose containing controls (Table 2).

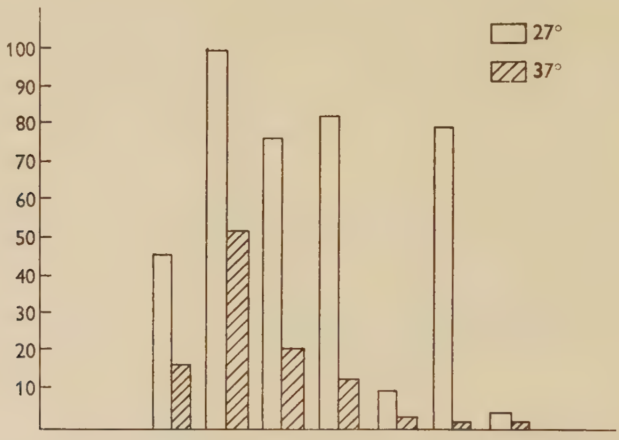

Fig. 1. Minimal amino acid requirements for pesticin biosynthesis by u.v.-irradiated *Pasteurella pestis* cells at 27° and 37°. Each column represents the average of ten experiments. The omission of L-lysine, glycine, L-valine, L-leucine, L-isoleucine, L-phenylalanine or L-methionine from the cysteine-supplemented MAA medium inhibited pesticin synthesis at either temperature.

L-Valine	+	+	+	+	+	+	+
L-Phenylalanine	+	+	+	+	+	+	+
L-Arginine	+	+	+	+	+	−	+
L-Methionine	+	+	+	+	−	+	−
L-Lysine	+	+	+	+	+	+	+
L-Leucine	+	+	+	+	+	+	+
L-Isoleucine	+	+	+	+	+	+	+
L-Alanine	−	−	+	+	+	−	−
L-Cysteine	−	+	+	−	−	+	+
Glycine	+	+	+	+	+	+	+

Amino acid requirement for growth of Pasteurella pestis, *Kimberley strain P*

Pasteurella pestis was grown on CHMG slants (CHMG plus 2 % (w/v) agar) at 27° for 24 hr. The bacteria were harvested and washed twice with phosphate buffer. Growth in amino acid media was determined by inoculating *P. pestis* into 3 ml. of medium in duplicate test tubes. The final concentration of the inoculum was 10^6 bacteria/ml. The tubes were incubated on a shaker for 72 hr. at 27° and growth was measured by count of colony formers. Growth of *P. pestis* was observed in the presence of six amino acids (methionine, phenylalanine, valine, isoleucine, cysteine and glycine). This medium did not support pesticin synthesis. Growth of *P. pestis* was observed also in the MAA medium. Addition of cysteine to this medium stimulated the bacterial growth (Table 3).

Table 2. *Pesticin biosynthesis in amino acid media with and without glucose addition*

Samples of 10^8 u.v.-irradiated *Pasteurella pestis* organisms/ml. were diluted 1/5 in the complete amino acid (CAA) medium and in the L-cysteine-supplemented minimal amino acid medium (CMAA), with and without 0·2 % (u/v) glucose. Pesticin synthesis was measured by pesticin titration after the 4 hr. incubation period. At zero time, no pesticin was detected.

Medium	Presence or absence of 0·2 % glucose	Incubation temperature (°C)	Pesticin conc. (arbitrary units/ml.)
CAA	+	27	500
CAA	–	27	750
CAA	+	37	500
CAA	–	37	500
CMAA	+	27	150
CMAA	–	27	100
CMAA	+	37	50
CMAA	–	37	50

Table 3. *Amino acid requirement for growth of* Pasteurella pestis *Kimberley strain P*

The organisms were inoculated on a basal amino acid medium containing the following amino acids: L-phenylalanine, L-valine, L-isoleucine and L-methionine. To this medium amino acids were added as indicated. Final concentration of the inoculum was 9×10^5 bacteria/ml. No growth was observed upon single addition of L-leucine, L-lysine, L-arginine or L-cysteine to the basal amino acid medium.

Amino acids added to basal amino-acid medium	Bacteria/ml. (after 72 hr.)
L-cysteine, glycine	$4{\cdot}1 \times 10^7$
L-leucine, L-lysine L-arginine, glycine: (MAA medium)	$6{\cdot}4 \times 10^7$
L-leucine, L-lysine, L-arginine, glycine, L-cysteine (CMAA medium)	1×10^8

Bacterial growth and pesticin synthesis

Freshly harvested and washed organisms in the logarithmic phase of growth were diluted to a concentration of about 10^8 bacteria/ml. and the suspension divided into two parts. One part was immediately suspended in an amino acid medium, and the growth was followed by colony count during incubation at 27° for 4 hr. The other part of the suspension was u.v.-irradiated and then suspended in the same medium; growth and pesticin synthesis were followed simultaneously during incubation at 27° for 4 hr. A typical experiment comparing growth and pesticin synthesis in CAA and MAA media is presented in Fig. 2. The rate of growth of non-irradiated bacteria was higher in the CAA medium than in the MAA medium. The rate of pesticin synthesis by irradiated bacteria was also higher in the CAA medium. The bacteria which survived irradiation grew more slowly in CAA medium than those which were not irradiated; in MAA medium the difference was not appreciable. In order to establish whether the bacteria irradiated in the stationary phase of growth are able to synthesize pesticin a culture of *Pasteurella pestis* was harvested after incubation for 3 days at 27°. Growth of the non-irradiated bacteria and growth and pesticin synthesis of the irradiated bacteria were measured. Though no growth of non-irradiated bacteria was detected by colony count in CAA medium, pesticin synthesis proceeded normally in the CAA and MAA media. A typical experiment is presented in Fig. 3.

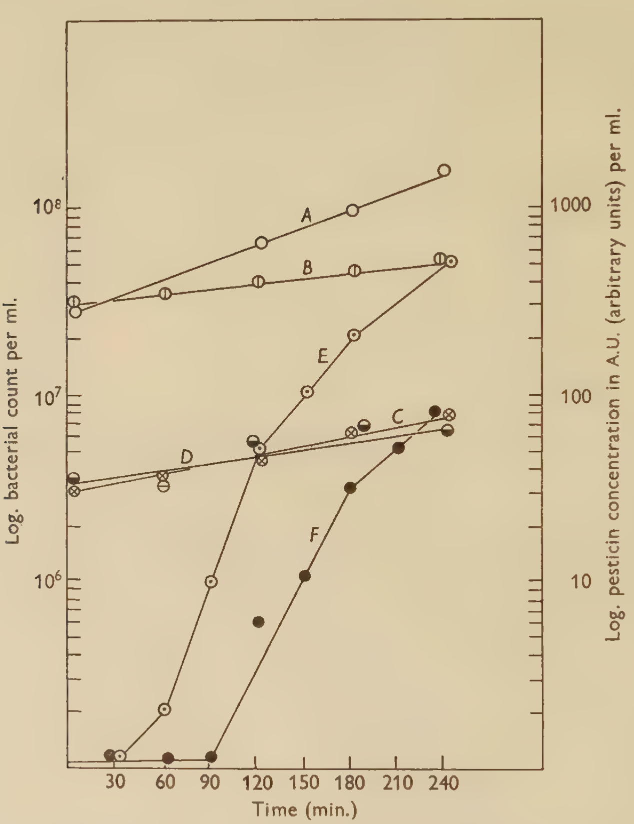

Fig. 2. Bacterial growth and pesticin biosynthesis by *Pasteurella pestis*, strain Kimberley P, in the logarithmic phase of growth in CAA and MAA medium at 27° and 37°. *A*, growth of non-irradiated bacteria in CAA medium; *B*, growth of non-irradiated bacteria in MAA medium; *C*, growth of irradiated bacteria in CAA medium; *D*, growth of irradiated bacteria in MAA medium; *E*, pesticin biosynthesis in CAA medium; F, pesticin biosynthesis in MAA medium.

The effect of chloramphenicol on pesticin synthesis

The effect of chloramphenicol, an inhibitor of protein synthesis (Gale & Folkes, 1953; Wisseman, Smadel, Hahn & Happs, 1954), on pesticin synthesis was tested. Irradiated *Pasteurella pestis* in the logarithmic phase of growth was suspended in CAA medium containing increasing concentrations of Synthomycetin U.S.P. (trade name for chloramphenicol; Abic, Ramat-Gan, Israel) and pesticin synthesis was measured after incubation at 27° and 37° for 4 hr. The effect of the same concentrations of chloramphenicol (which was below the concentration which caused bacteriostasis) on the growth of non-irradiated bacteria during the same incubation period at 27° and 37° was determined. Control measurements of growth and pesticin synthesis in CAA medium were performed. The results are shown in Table 4. It can be seen that similar concentrations of chloramphenicol inhibited both the growth of non-irradiated bacteria and pesticin synthesis by the irradiated bacteria. Chloramphenicol

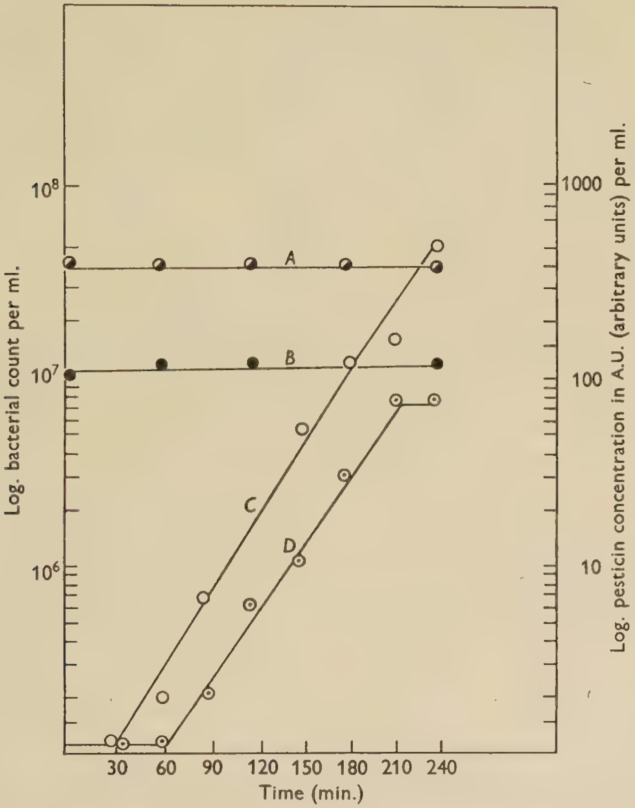

Fig. 3. Bacterial growth and pesticin biosynthesis by *Pasteurella pestis*, strain Kimberley P at the end of the logarithmic phase of growth in CAA and MAA medium at 27° and 37°. *A*, growth of non-irradiated organisms in CAA medium; *B*, growth of irradiated organisms in CAA medium; *C*, pesticin biosynthesis in CAA medium; *D*, pesticin biosynthesis in MAA medium.

at 1 μg./ml. exerted a markedly inhibitory effect on pesticin synthesis at 27° and 37°.

Table 4. *Comparison of inhibitory effect of graded low chloramphenicol concentrations on: (a) pesticin biosynthesis by irradiated* Pasteurella pestis; *(b) the growth of non-irradiated bacteria*

Samples of u.v.-irradiated *P. pestis* 10^8 bacteria/ml. were diluted 1/5 in CAA medium containing low chloramphenicol concentrations and pesticin biosynthesis after 4 hr. incubation was measured. Growth of non-irradiated bacteria at the same chloramphenicol concentrations was determined by colony count. CAA medium without chloramphenicol was used for control determination of pesticin yield and bacterial growth.

	Irradiated *P. pestis* Incubation temperature		Non-irradiated *P. pestis* Incubation temperature	
	27°	37°	27°	37°
Chloramphenicol (μg./ml.)	Pesticin yield (% of control)		Bacterial growth (% of control)	
0·0	100	100	100	100
0·5	100	100	100	100
1·0	44	33	100	83
2·0	25	7	74	46
3·0	10	0	47	36

Non-pesticinogenic mutants of Pasteurella pestis

To test whether non-pesticinogenic mutants of the Kimberley strain of *Pasteurella pestis* could be obtained, the following experiments were performed. *P. pestis* (streptomycin-resistant) grown in Pr3 broth to a concentration of 10^8 bacteria/ml. were u.v.-irradiated for 5 min. This dose killed 95 % of the organisms. The irradiated bacteria were plated on Pr3A plates and tested for pesticinogenic properties by the double layer method (Ben-Gurion & Hertman, 1958). Streptomycin-sensitive *P. pseudotuberculosis* was used as indicator. Colonies which did not produce pesticin were removed with a bacteriological loop and streaked on BAB medium containing 500 μg. streptomycin/ml. The developed colonies were streaked once more on streptomycin-containing BAB medium. The strains thus obtained were tested for rhamnose fermentation, pesticin production and pesticin sensitivity. Three independent mutants were isolated by plating 10^5 bacteria. These mutants were streptomycin-resistant and did not ferment rhamnose and were thus distinguished from the *P. pseudotuberculosis* indicator strain. These non-pesticinogenic mutants were sensitive to pesticin.

DISCUSSION

A defined amino acid medium has been developed which supports pesticin production by u.v.-irradiated *Pasteurella pestis*. In view of the requirement for amino acids and the sensitivity of the system to chloramphenicol, bacteriocinogenesis by *P. pestis* may represent a *de novo* protein synthesis.

Experiments thus far have not revealed whether the described amino acid requirements are specific for the synthesis of pesticin or represent general requirements for protein synthesis by the bacterial cell. A higher yield of pesticin was obtained in the CAA and CMAA media than in the MAA medium.

The former media also supported a higher rate of growth of non-irradiated bacteria. These observations indicate that the amino acids may be required for the general synthetic activity of the bacteria and only indirectly influence pesticin synthesis. Further proof of the dependence of pesticin synthesis upon the general synthesizing capacity of the bacterial cell may be found in the observation that maximum pesticin production occurred in a medium which supported abundant bacterial growth from a small inoculum and which thus could replace more complex media, as reported by other authors (Rockenmacher, James & Elberg, 1952). On the other hand, it may be concluded that the amino acid requirements described in this work are specific directly for pesticin synthesis, since the latter was observed also in bacteria in the stationary phase of growth. Also, *Pasteurella pestis* can be grown in a defined medium containing six amino acids (phenylalanine, valine, methionine, isoleucine, cysteine and glycine), which does not support pesticin synthesis. No decision can yet be reached between these two alternatives. Media in which maximum pesticin synthesis was not reached gave a lower production at 37° than at 27°. These observations indicate more efficient protein synthesis at 27° than at 37°. The amino acids supplied to the defined media were an adequate source of energy, and no addition of glucose was needed. Mutants of *P. pestis* which lost the pesticinogenic property became sensitive to the pesticin they originally produced. A similar behaviour has been observed for the colicinogenic strains of *Escherichia coli* (Fredericq, 1948).

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Taxonomic Studies of the Genus *Clostridium*: *Clostridium bifermentans* and *C. sordellii*

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SUMMARY: In an attempt to elucidate the taxonomic relationship of *Clostridium bifermentans* and *C. sordellii* about sixty strains assigned to one or other of the two species were examined. Two groups of strains differing in their cultural and biochemical properties were recognized. One closely resembled the type culture of *C. bifermentans* (Tissier & Martelly, 1902), and the other, in which some strains were pathogenic, resembled the *C. sordellii* isolate '82' of Sordelli. The former of these groups is clearly to be regarded as *C. bifermentans* and the latter as *C. sordellii*. It has been shown that the two species, although similar in many respects, are distinct and easily distinguishable. In view of this, the present practice of some bacteriologists of combining the two species under the name *C. bifermentans* is incorrect and should be discontinued.

Although *Clostridium bifermentans* was first isolated by Tissier & Martelly in 1902, and *C. sordellii* by Sordelli in 1922, the taxonomic relationship of these two organisms is still the subject of controversy. The use of either of two criteria (cultural and biochemical character or pathogenicity) for the separation of *C. bifermentans* and *C. sordellii*, together with the failure of many American and British bacteriologists to recognize their existence as separate species, has given rise to much confusion. In the English-language literature most authors combine the two species under the name *C. bifermentans* (noting the existence of *C. sordellii* as a pathogenic variety) and quote a description including features of the two organisms only some of which are common to both. Most French workers recognize the existence of the two species, mainly on serological grounds, but the evidence they present is based on the examination of very few *C. sordellii* strains.

To confuse the matter further, each species has been described under at least three names. The original isolate of *Clostridium bifermentans* was named *Bacillus bifermentans sporogenes* (Tissier & Martelly, 1902) and later re-named *B. bifermentans* (Weinberg & Séguin, 1918), in accordance with the principle of binominal nomenclature. In 1922 Hall examined a number of alleged *B. bifermentans* strains and found some to be non-motile (as in the original description) and others to be motile; for the latter he proposed the creation of a new species, *B. centrosporogenes*. Consequent on the demonstration of motility in young cultures of *B. bifermentans* (Levenson, 1936), the two species were recombined (McCoy & McClung, 1936; Clark & Hall, 1937). *C. sordellii* was originally named *B. oedematis sporogenes* (Sordelli, 1922), and was re-named *B. sordellii* by Hall & Scott in 1927. In the same year Meleney, Humphreys & Carp (1927) isolated an anaerobic bacillus which they considered to be a new species and named *B. oedematoides*, and which was later shown to be identical with *B. sordellii* (Humphreys & Meleney, 1927–8; Hall & Scott, 1931).

The first suggestion that *Clostridium bifermentans* and *C. sordellii* might be regarded as one species appears to have been made by Stewart (1938) on cultural and serological grounds, although in 1937 Clark & Hall had already demonstrated cross-agglutination between the two species, and noted the ability of *C. bifermentans* antisera to confer some protection against challenge with *C. sordellii*. The latter authors, however, considered that *C. sordellii* merited species status because of its marked pathogenicity. Lillie (footnote, 1938) referred to the work of Clark & Hall and of Stewart, but did not think that the two species had been fully established as identical. Since Stewart's paper of 1938, the concept of *C. bifermentans* and *C. sordellii* as a single species seems to have been generally accepted by British and American workers, including Smith (1955), and the authors of the relevant sections of *Bergey's Manual* (1957), *Topley and Wilson's Principles* (1955) and Reed (1952). However, Sterne & van Heyningen, in *Bacterial and Mycotic Infections of Man* (1958), considered *C. bifermentans* and *C. sordellii* to be distinct, but not necessarily worthy of separate species status. Prévot (1938), in a taxonomic study of the genus *Clostridium*, described *C. bifermentans* and *C. sordellii* under separate headings. Later, Prévot & Cordier (1941) studied the metabolism of the two species, and, although they found them to be virtually identical in this respect, considered that the pathogenicity of *C. sordellii* justified its species status. In 1952 Huet & Aladame, using the urease test of Roland, Bourbon & Szturm (1947), noted the production of urease by *C. sordellii*. Tardieux & Nisman (1952), as a result of serological studies of extracts of *C. bifermentans* and *C. sordellii* cultures, concluded that the species were distinct although immunologically closely related. Tataki & Huet (1953) examined a number of *C. bifermentans* and two *C. sordellii* strains serologically and for urease production. They were able to distinguish two serological groups, one comprising all the urease-positive strains including some which had been assigned to *C. bifermentans* (presumably on the grounds of non-pathogenicity), and the other consisting of the urease-negative *C. bifermentans*, together with two strains of the latter species in which the urease reaction was doubtful. They concluded that all the urease-positive strains, including those lacking pathogenicity, should be regarded as *C. sordellii*. This concept of *C. sordellii* and *C. bifermentans* as two distinct species, the former characterized by urease production and variable as regards pathogenicity, and the latter non-urease producing and non-pathogenic, is upheld by Prévot (1955). Prévot (1957) described *C. sordellii* as rods, 3·0–5·0 μ by 1·2–1·5 μ; motile; forming arborescent colonies in deep agar; fermenting glucose, fructose, and maltose, but not glycerol or sorbitol; pathogenic; highly toxigenic; and producing urease. *C. bifermentans* was described as rods, 5·0–6·0 μ by 0·8–1·0 μ; motile; forming clustered lenticular or raspberry-like colonies in deep agar; fermenting glucose, fructose, maltose, glycerol and sorbitol; non-pathogenic; non-toxigenic; and non-urease producing. Prévot also stated that *C. sordellii* was more strongly proteolytic than *C. bifermentans* although protein metabolism followed the same pattern in both species; that neither species produced nitrites from nitrates; and that some strains from each were haemolytic and produced lecithinase. Recently Meisel & Rymkiewicz (1958), investigating the antigenic properties of spores of proteolytic clostridia, found no trace of cross-agglutination between *C. sordellii* and *C. bifermentans* spore suspensions and spore-antisera.

The investigations reported below were undertaken to elucidate the relationship of *Clostridium sordellii* and *C. bifermentans* in the course of general taxonomic study of the genus *Clostridium* (as defined in *Bergey's Manual*, 1957).

METHODS

Strains examined

Sixty-two cultures obtained from various sources were studied. To simplify the tabulation and discussion of results the strains will be grouped under three headings:

Clostridium bifermentans: those strains which are non-pathogenic and non-urease producing, resembling the type strain (Tissier & Martelly). Twenty-four strains were examined; National Collection of Type Cultures, nos. 1341, 506 and 6928; Wellcome Research Laboratories Collection, nos. 1617, 2086, 1622, 1625, 1626 and 1628; Institut Pasteur, Paris, Collection, 'TM'; L. DS. Smith's Collection, nos. B-5, B-11, B-13, B-14, B-26, B-27, S-6479, S-7172 and U-VRL; and five strains isolated at the Wellcome Research Laboratories, 'Bif. 1, 2 and 3' from soil, 'OWM 2' from pathological material received from Khartoum, and '10015R' from a contaminated culture of *C. multifermentans.*

Clostridium sordellii (P): those strains which are pathogenic and produce urease. Thirteen strains were examined; National Collection of Type Cultures, nos. 8780 and 2919; Wellcome Research Laboratories Collection, nos. 1732, 1733, 1734 and 1694; L. DS. Smith's Collection, no. S.-15; Mr J. Hepple's Collection, '492E'; and five strains isolated at the Wellcome Research Laboratories, nos. 646L and 650L from contaminated cultures of *C. sphenoides*, '46/296' from a culture received from Iran, and nos. 83050 and T isolated from a dead heifer.

Clostridium sordellii (N-P); those strains which produce urease but are non-pathogenic. Twenty-five strains were examined; National Collection of Type Cultures, nos. 1340, 6927, 6801, 6800, 2914, 6929, 6799 and 6801 (opaque variant); Wellcome Research Laboratories Collection, nos. 1619, 1620, 1621, 1623, 1624, 1627 and 3936; L.DS. Smith's Collection, nos. S-7048E, S-7166, S-7363, S-8841, S-8853 and B-AA5; Mr J. Hepple's Collection, nos. 484E and 512E; and two strains '9831' and '9833' isolated from pathological material at the Wellcome Research Laboratories.

Fermentation reactions

Media. Fermentation of carbohydrates was examined in a peptone water medium containing the carbohydrate under test at 1·0 % (w/v) concentration, and 0·2 % (v/v) of a 6·0 % (w/v) solution of bromocresol purple. The medium was dispensed in $\frac{1}{4}$ oz. screw-cap bottles, and iron strip added to promote reducing conditions. The carbohydrates used for the tests were arabinose, rhamnose, xylose, glucose, fructose, galactose and mannose; lactose, sucrose, maltose and trehalose; raffinose, inulin, dextrin, glycogen and starch; glycerol, adonitol, mannitol, sorbitol, dulcitol and inositol; salicin and aesculin.

Inoculation and incubation. The peptone water sugars were inoculated with 24 or 48 hr. Robertson's meat broth cultures, and incubated at 37° for 2 weeks.

Method of testing. Cultures were examined for the production of acid and gas, Universal Indicator (British Drug Houses Ltd., Poole) being added where the bromocresol purple had been decolorized. The pH value of cultures in aesculin peptone water were checked potentiometrically, and a reading of less than pH 6·5 was recorded as positive for acid production.

Proteolytic reactions

To demonstrate proteolytic activity strains were grown in media containing, respectively, gelatin, casein, coagulated serum, azocoll (Oakley, Warrack & van Heyningen, 1946), meat particles and coagulated egg albumin.

Titration of gelatinase activity. The substrate used was a 10 % (w/v) solution of gelatin in distilled water, with the addition of Congo red, and 0·5 % phenol as preservative. The reagent was stored in the cold and melted by warming to about 40° immediately before use.

Doubling dilutions of samples of 48 hr. cultures were added in 1·0 ml. amounts to 1·0 ml. of the gelatinase reagent. After thorough mixing, as shown by the even distribution of the Congo Red colour, the mixtures were incubated at 37° for 18 hr., then placed at 4° for 1 hr., and the results read at this temperature.

Chromatographic examination of Clostridium bifermentans *and* C. sordellii *cultures*

Twenty-five ml. volumes of the chromatographic medium of Woiwod & Proom (1950) and of Robertson's meat broth were used for this study. Both broth and agar surface cultures were used as inocula. Incubation was at 37° (in an anaerobic jar in the case of the chromatographic medium) and the cultures were tested after 1, 3 and 7 days.

The solvent used for chromatography was butanol + acetic acid, and the chromatograms were sprayed with ninhydrin for general examination, and with Pauley's diazo reagent for histidine and related compounds.

Urease production

Cultures were grown for 24 and 48 hr. at 37° in 20 ml. amounts of Robertson's meat broth. A few strains from each group were also grown in 100 ml. amounts of a similar medium and tested daily for 4 days.

The reagent used was a slightly modified version of that of Roland, Bourbon & Szturm (1947) and Huet & Aladame (1952), and was made up as follows: 0·1 g. KH_2PO_4; 0·1 g. K_2HPO_4; 0·5 g. NaCl; 2·0 g. urea; 1·0 ml. 95 % (v/v) ethanol in water; 100 ml. distilled water; 5·0 ml. Universal Indicator; sufficient 0·1 N-HCl to give an orange colour (about pH 6·0).

A 2·5 ml. sample of the culture under test was transferred to a test tube (80 mm. × 8·0 mm.) and centrifuged. The supernatant fluid was discarded and the sediment washed once in 1·0 % (w/v) saline and finally resuspended in 1·0 ml. distilled water. One ml. of urease reagent was added and, after thorough mixing, the test was incubated at 37°.

Various biochemical reactions

Indole production. Twenty-four- and 48-hr. cultures in Robertson's meat broth were examined for indole production by means of Ehrlich's reagent and by the vanillin test (Spray, 1936).

Bromocresol purple milk. Cultures were incubated at 37° for 1–14 days.

Nitrite production. All strains were grown in nitrite-free broth containing potassium nitrate and incubated at 37° for 2–3 days. Cultures were examined for the presence of nitrites by means of sulphanilic acid and diphenylamine in acetic acid.

Voges-Proskauer and Methyl Red tests. All strains were grown for 24 and 48 hr. in glucose phosphate broth, and tested for the production of acetylmethylcarbinol by O'Meara's modification of the Voges-Proskauer test, and for acid production by the addition of methyl red.

Toxin production and pathogenicity

Lecithovitellin plates. All strains were grown on agar plates containing lecithovitellin (Macfarlane, Oakley & Anderson, 1941) with or without the addition of *Clostridium welchii* α-antiserum. Incubation was in anaerobic jars at 37° for 24 hr.

Blood agar plates. All strains were grown on horse-blood agar plates incubated in anaerobic jars at 37° for 24 hr.

Haemolysin and lecithinase production in broth cultures. All strains were examined after growth for 6 and 24 hr. in 100 ml. volumes of broth containing meat particles. Thiomersalate at 0·01 % (w/v) concentration was added to all cultures before testing.

Buffer solutions. The buffer solutions used were saline containing $CaCl_2$ and gelatin ('cagsaline'), cagsaline with the addition of *Clostridium welchii* antiserum to give a final concentration of 5·0 α units/ml., borate buffer saline, 'phosthio' buffer (see below) and sodium acetate buffer.

'*Cagsaline*' (modified from Oakley & Warrack, 1941): add 138·75 ml. of 2·0 % (w/v) calcium chloride solution to 100 ml. of 5·0 % (w/v) gelatin solution and 22·5 g. sodium chloride; make up to 2·5 l. with distilled water, add 0·2 g. thiomersalate and 2·5 g. phenol, fill out and autoclave at 10 lb./sq.in. for 10 min.; keep in cold.

Borate buffer saline (modified from Glenny, Pope & Waddington, 1928): dissolve 30 g. of a mixture of 59 g. borax +84 g. boric acid +99 g. sodium chloride in 2 l. 0·3 % (w/v) sodium chloride solution.

'*Phosthio*' *buffer* (Oakley, Warrack & Clarke, 1947): to 4·0 ml. distilled water add 0·1 ml. thiolacetic acid, adjust pH to 6·5 with N-NaOH, and make up to 25 ml. with M/5 phosphate buffer (pH 6·5).

Sodium acetate buffer (Oakley, Warrack & Clarke, 1947): to 27·2 g. sodium acetate in 1 l. distilled water add 50 ml. of 5·0 % (v/v) acetic acid in 1·0 % saline.

Procedure. One ml. of culture was added to 0·5 ml. of each of the five buffers; these mixtures were made up in triplicate. To one set of mixtures was added 0·5 ml. of a 1/10 suspension of sheep red blood cells, to another 0·5 ml. of a 1/10 suspension of horse red blood cells, and to the third 0·5 ml. of lecithovitellin (a suspension of egg-yolk in saline). After thorough mixing all tests were incubated in a water bath at 37° for 2 hr. and then left at room temperature for 16 hr. after which the results were read.

Animal tests

Media and incubation. All strains were inoculated into 100 ml. amounts of broth containing meat particles and incubated at 37° for 24 hr. Some strains from each group were also tested after incubation for 6 hr. Thiomersalate to 0·01 % (w/v) final concentration was added to all filtrates and supernatant fluids, but not to cell suspensions.

Intravenous injections. Culture filtrates were injected intravenously into mice in 0·5 ml. amounts. Strains which failed to kill were retested using supernatant fluids from centrifuged cultures. Filtrates and supernatant fluids were injected very slowly since both contained some factor which caused profound shock or death on rapid intravenous injections.

Intradermal injections. Culture filtrates, undiluted and diluted 1/5 and 1/25 in broth were injected intradermally into guinea-pigs in 0·25 ml. amounts. The results of the injections were read after 1 and 2 days.

Intramuscular injection. Suspensions of twice-washed bacteria in 1·0 % saline containing 0·5 % (w/v) calcium chloride were injected into the hind legs of mice in 0·5 ml. amounts. All dead mice were examined for the characteristic signs of *Clostridium sordellii* infection.

Morphology of colonies and bacteria

Morphology of colonies. All strains were grown on horse-blood agar plates containing 1·8 % (w/v; normal) and 3·0 % (w/v; stiff) New Zealand agar, and containing 5·0 % (v/v) horse blood. Inoculated plates were incubated in anaerobic jars for 24 and 48 hr.

Morphology of bacteria. Twenty-four- and 48-hr. agar surface and broth cultures were examined. Measurements of the size of the bacteria were made on heat-fixed Gram-stained films using a grid eye-piece. Films stained by Giemsa's, Muir's, and the Alcian-blue + carbol fuchsin and phosphomolybdic acid + methyl green methods were also examined

RESULTS

Fermentation reactions

The results of the fermentation tests are shown in Table 1. The fermentation of mannose, sorbitol and salicin clearly differentiated the *Clostridium bifermentans* strains from those of the two *C. sordellii* groups, although one *C. bifermentans* strain failed to fement sorbitol, and another salicin. Aesculin was fermented by only half of the *C. bifermentans* strains and was therefore less useful for differentiation. The present results are not wholly in agreement with many reported elsewhere. Many discrepancies are because some workers have regarded *C. bifermentans* and *C. sordellii* as a single species, and because others have used pathogenicity as the sole criterion for species separation, and thus including urease-positive strains in *C. bifermentans*. The production of acid and gas from glycerol by all but two of the *C. sordellii* strains (those which produced gas only) was contrary to the observations of Prévot and of several other workers; no explanation can be suggested for this discrepancy.

Table 1. *Results of fermentation tests*

		No. of strains fermenting								
Group	No. of strains examined	Glucose	Fructose	Mannose	Maltose	Glycerol	Sorbitol	Salicin	Aesculin	Other sugars
C. bifermentans	24	24	24	24	24	24	23	23	16	0
C. sordellii (P)	13	13	13	0	13	13	0	0	0	0
C. sordellii (N–P)	25	25	25	0	25	25*	0	0	0	0

* Two strains repeatedly produced abundant gas but no acid in glycerol-peptone water.

Proteolytic reactions

All strains digested casein, coagulated serum, azocoll, meat particles and, to a lesser extent, coagulated egg albumin which was blackened by all strains but only completely digested by a few.

Gelatinase activity. All strains liquified gelatin and a rough quantitative measurement of gelatinase activity was made in an attempt to confirm Prévot's observation that *Clostridum bifermentans* produced less gelatinase than *C. sordellii*. The results are shown in Table 2. The results obtained did not suggest a constant difference between the three groups in respect of gelatinase activity, although the *Clostridum bifermentans* strains tended to produce the most gelatinase. However, this group showed the widest variation in gelatinase production and included some of the least active strains.

Chromatographic examination of cultures

Grown in chromatographic medium. No consistent difference between the groups was seen. This agreed with the findings of Prévot & Cordier (1941).

Grown in Robertson's meat broth. Paper chromatography revealed distinct and consistent differences between the *Clostridium bifermentans* and the *C. sordellii* cultures. The most obvious difference was the absence of histidine from the *C. bifermentans* cultures and its presence in large amounts in both pathogenic and non-pathogenic *C. sordellii.*

Table 2. *Production of gelatinase by* Clostridium bifermentans *and* C. sordellii *strains. Results of gelatinase titrations*

Group	No. of strains examined	No. of strains positive at dilution								
		1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
C. bifermentans	24	23	22	19	18	16	13	10	3	0
C. sordellii (P)	13	13	13	12	6	0	0	0	0	0
C. sordellii (N–P)	25	23	19	12	4	0	0	0	0	0

Urease production

The speed at which positive reactions developed varied with the amount of urease produced. All the urease-producing strains gave positive results within 4–5 hr., although the strength of the reactions increased up to 48 hr., with colour changes through yellow-green, green and blue-green to blue in strongly positive strains. Tests negative at 5 hr. were still negative at 72 hr. All strains gave unequivocal results which were consistently reproducible; replicate cultures of any strain gave virtually the same final colour. In the urease positive strains (by definition *Clostridium sordellii*) no correlation was observed between pathogenicity and the strength of the urease reaction. An unexpected observation made in the course of these tests was that many *C. bifermentans* strains had the ability to suppress the positive urease reactions when grown as mixed cultures with *C. sordellii.* This inhibition was confirmed on several occasions and might easily mask the presence of *C. sordellii* if the urease reaction were used as a 'diagnostic' test with a naturally occurring mixture of the two species. No inhibition was observed when washed sediments or supernatant fluids of *C. bifermentans* cultures were added to *C. sordellii* sediments immediately before testing. As indicated in Table 1, the subdivision of the *bifermentans/sordellii* complex on the basis of urease production agreed with subdivision based on fermentation reactions.

Various biochemical reactions

Indole production. All strains in each of the three groups produced indole, although six of the twenty-four strains of *Clostridium bifermentans* gave very weakly positive reactions. A number of strains in each group were also tested after growth for 8 days; all the *C. bifermentans* cultures gave negative, and the *C. sordellii* cultures positive reactions with Ehrlich's reagent and the vanillin test. None of the strains gave a vanillin violet reaction.

Bromocresol purple milk. All strains tested clotted and then digested the milk. The speed and extent of digestion of the clot, decolorization of the indicator and the amount of acid produced were variable but apparently uncorrelated with urease production.

Nitrite production. All strains gave negative results when tested for nitrite.

Voges-Proskauer and Methyl red tests. No strain produced acetylmethylcarbinol. The results of the Methyl red test were variable: 18 of the 24 cultures of *Clostridium bifermentans*, 8 of the 13 cultures of *C. sordellii* (P) and 19 of the 23 cultures of *C. sordellii* (N-P) gave positive results.

Toxin production and pathogenicity

Lecithovitellin plates. Most strains in each group produced weak to moderately strong precipitation, which was in all cases inhibited by the addition of *Clostridium welchii* α-antiserum. No colonies were lustrous.

Blood agar plates. Most cultures of each of the three groups were haemolytic, although in some the degree of haemolysis was very slight and did not extend beyond the edges of the colonies. A few strains in each group were consistently non-haemolytic.

Haemolysin and lecithinase production in broth cultures. There was little or no difference between the groups in respect of the production of haemolysins and lecithinases. None of the *Clostridium bifermentans* strains haemolysed sheep cells in any of the buffers, but apart from this observation, which requires further investigation, the results generally confirmed the observations of Miles & Miles (1947), Macfarlane (1948), Prévot & Capponi (1949) and Prévot & Malgras (1950) that some strains of each species produce haemolysins and lecithinases which are immunologically related to the α-lecithinase of *C. welchii.* No differences between the three groups were observed.

Animal tests. None of the urease-negative cultures (*Clostridium bifermentans*) showed any lethal, dermonecrotic or pathogenic properties. Thirteen of the urease-positive strains (*C. sordellii* (P)) were rapidly lethal on intravenous injection, elicited the production of characteristic *C. sordellii* skin lesions (central purple-brown necrotic area with surrounding and underlying massive oedema), and caused death within 18 hr. when injected intramuscularly. The remaining twenty-five urease-positive strains (*C. sordellii* (N–P)) showed no lethal, demonecrotic or pathogenic activity, and were indistinguishable by animal tests from the urease-negative strains. The fact that strongly lecithinase-positive 6 hr. culture filtrates of *C. bifermentans* and *C. sordellii* (N–P) strains were non-toxic agreed with the observations of Miles & Miles (1947) that the lecithinases of these species are considerably less toxic than the immunologically closely related α-lecithinase of *C. welchii.*

Morphology of colonies and bacteria

Morphology of colonies. The colonies of *Clostridium sordellii* and *C. bifermentans* were frequently so dissimilar that it would have been possible to assign most strains to their correct species on appearance alone. On normal plates (1·8 % (w/v) New Zealand agar) even after thorough drying, the *C. sordellii* colonies tended to spread, forming a continuous film with a characteristic marbled pattern. On stiff plates (3 % (w/v) New Zealand agar) they tended to form lozenge-shaped colonies, the long axes of which followed the direction of streaking. These colonies were greyish white, translucent in young cultures but became more opaque with the onset of spore formation, with crenate to coarsely rhizoidal margins, and peaked and ridged contours. The *C. bifermentans* strains formed opaque, white to yellowish, more or less circular, low convex or flat-topped colonies with entire to slightly undulate margins. They showed no tendency to spread except on very wet normal agar plates. The differences between *C. bifermentans* and *C. sordellii* colonies are shown in Pl. 1.

Morphology of bacteria. No morphological differences between the bacteria of the three groups were observed. Rods of all strains were within the size range *c.* 0·6–1·0 μ width by 2·0–6·0 μ length, and all were strongly Gram-positive. There was no difference between groups in respect of the occurrence, shape, position or size of spores, which were cylindrical to oval, central to subterminal, and did not noticeably swell the rods.

DISCUSSION

In the light of the investigations detailed above, and of the findings of other workers, particularly those reported in the *Annales de l'Institut Pasteur*, it is clear that two distinct groups, differing consistently in respect of a number of characters, exist within the *bifermentans/sordellii* complex. The only question, therefore, is whether these differences are sufficiently great to merit the raising of the groups to species status. The criteria for species separation within the Eubacteriales, particularly in the case of the pathogenic organisms, are somewhat variable. However, it is difficult to justify the inclusion within the same species of organisms differing in colony morphology, fermentation of carbohydrates, protein metabolism and urease production, especially when these differences are correlated with serological groupings (Tataki & Huet, 1953; Tardieux & Nisman, 1952; Meisel & Rymkiewicz, 1958). The separation of *Clostridium bifermentans* from *C. sordellii* (using urease production and fermentation reactions as criteria) would therefore seem inevitable. The question then arises as to the validity of using pathogenicity for subdividing the strains assigned to *C. sordellii*. Pathogenicity, unsupported by other characteristics, is not a satisfactory criterion for species separation, since it is frequently lost, especially after repeated subcultivation. There are several known non-pathogenic subcultures derived from Sordelli's presumably originally pathogenic isolate '82'. Thus there appears to be scant justification for granting separate species status to the pathogenic and non-pathogenic urease-positive strains, and the inclusion of both within *C. sordellii* (as suggested by Tataki & Huet, 1953, and Prévot, 1955, 1957), would not prejudice further subdivision if future research showed this to be desirable.

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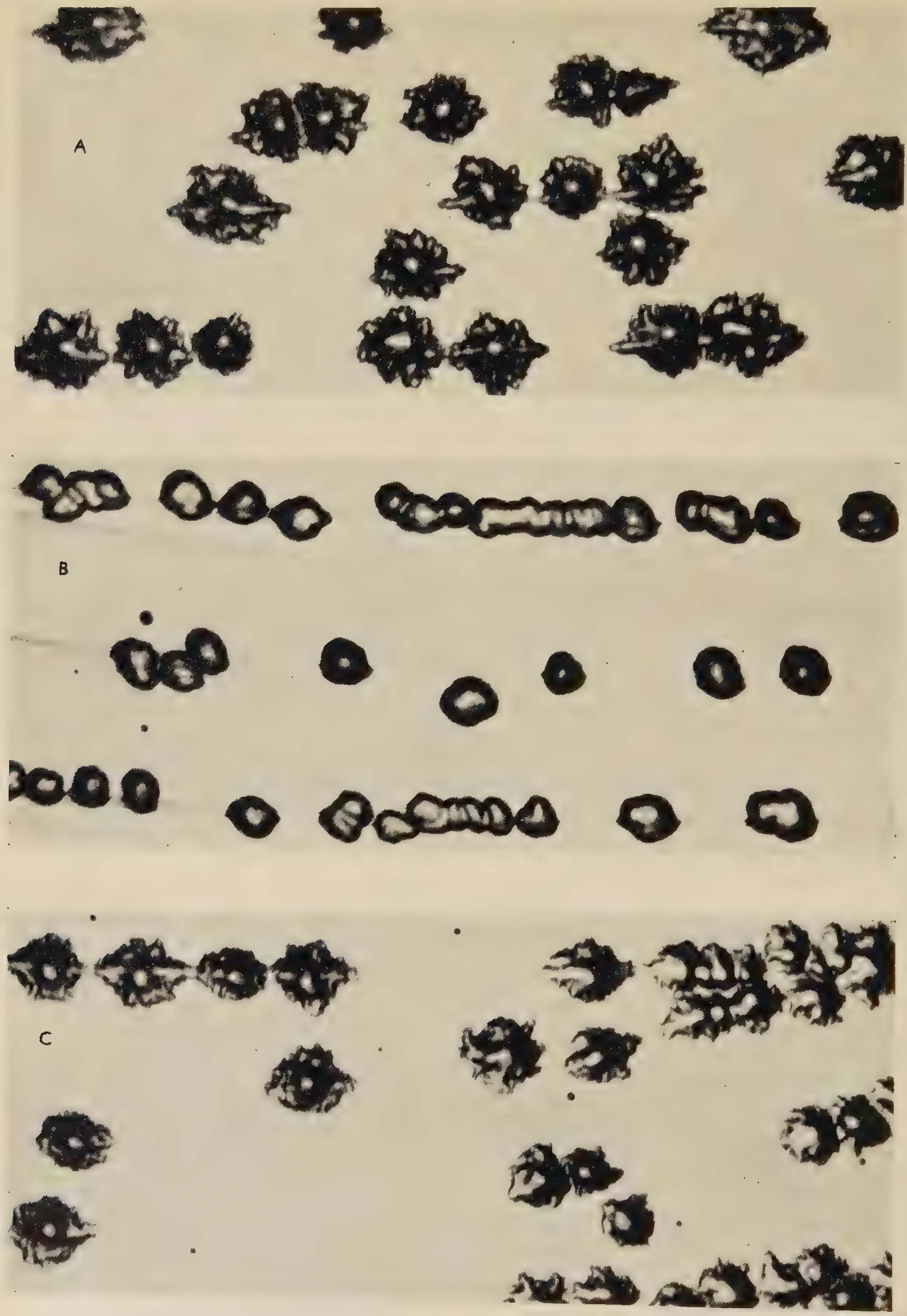

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EXPLANATION OF PLATE

Colonies of *Clostridium sordellii* and *C. bifermentans* on stiff horse-blood agar plates. A, *C. sordellii* (pathogenic); B, *C. bifermentans*; C, *C. sordellii* (non-pathogenic). (All × 6.)

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Gel Diffusion Studies on Serotype and Serotype Transformation in Paramecium

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SUMMARY: Antigens G and E of stock 28 variety 2 of *Paramecium aurelia* show cross-reactions with their respective antisera in immobilization and gel diffusion tests. In immobilization tests the anti-E serum P, no. 132, shows the strongest cross-reaction; in gel diffusion tests the anti-G serum P, no. 130, cross reacts more strongly. During serotype transformation of type G protozoa to type E, the increase of the E antigen and the disappearance of the G antigen were followed by the gel diffusion technique. However, because of the peculiar cross-reactions of these two antigens, only the increase of the new (E) antigen was studied quantitatively by this technique. No reliable measurements of the loss of the old (G) antigen were obtained. The results indicate that during transformation the amount of the new antigen increased exponentially.

Within a given stock of *Paramecium aurelia* a number of clones representing different antigenic types or serotypes may be obtained. Each clone is immobilized by an appropriate dilution of homologous antiserum made against protozoa of its own serotype and unaffected by similar dilutions of sera made against other serotypes. Studies of the immobilization antigens of Paramecium by Sonneborn and others have revealed that they are determined by a complex interaction of three factors: the environment, a system of cytoplasmic inheritance, and nuclear genes (review; see Beale, 1957). Changes in the environment often induce a transformation from one serotype to another. Often the different serotypes of a stock are stable under the same environmental conditions. Under such circumstances crosses between the different serotypes reveal that the differences are maintained by a system of cytoplasmic inheritance. The serotypes which can be manifested, their specific properties and also their stability have been shown to vary from stock to stock, and to be under the control of nuclear genes.

The data are consistent with the idea that there is a separate primary genic locus for each serotype, a locus which influences whether the serotype in *Paramecium aurelia* can be expressed, its stability, and its immunological specificity. More than one locus, however, may be involved in stability. Normally, the effect of only one primary locus is evidenced at any one time, i.e. only one antigen is detectable. In most of the cases studied any one of a number of equally competent loci may come to expression under a particular set of conditions. Once a type is expressed, however, the continued expression of that locus rather than the others is transmitted hereditarily through the cytoplasm. The expression of one locus may be suppressed and another allowed to be manifested by changing the conditions of culture.

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The phenomenon of transformation of protozoa of one serotype into another is of particular interest from the point of view of gene action during development and differentiation. Upon transformation the action of a given locus ceases to be manifested and the effect of another appears. During the period of transition (about two fissions) both the disappearing serotype and the new one coming to expression may be detected at the same time (Beale, 1948, 1954; Margolin, 1956).

Studies of the transformation process have been hampered until recently by the lack of a quantitative method for assay of the antigen. This difficulty was overcome by Finger (1956) who used a technique of precipitation in a gel. Gel-diffusion techniques provide a qualitative and quantitative method of measuring antigens in complex mixtures (Oudin, 1952; Oakley & Fulthorpe, 1953; Preer, 1956; Preer & Telfer, 1957). Finger found that while protozoa of different serotype contained many common antigens, each immobilization serotype had a unique antigen not found in any of the others. Preer & Preer (1959) studied a series of antigens obtained from protozoa of one serotype, designating them antigens 1, 2, 3 and 4. Antigen 4 was the serotype correlated antigen described by Finger. As shown by Finger, there are a number of serologically different antigens 4, each corresponding to a specific immobilization serotype. It is assumed that these antigens are the immobilization antigens, and they will be referred to as antigens A, B, C, etc., corresponding to the serotypes from which they are obtained. Preer & Preer (1959) reported that antigen G of variety 2 of *Paramecium aurelia* has the properties of a protein and is localized in the cilia and pellicle. Beale & Kacser (1957), using fluorescent antibody, reached similar conclusions about the site of the immobilization antigens in variety 1.

The present paper reports the results of a preliminary study of the kinetics of serotype transformation in *Paramecium aurelia* by using gel diffusion quantitatively to assay antigen. The transformation studied was from serotype G to serotype E in stock 28 of variety 2 of *P. aurelia* (Finger, 1957*a*). The two serotypes proved to cross-react rather strongly in gel diffusion and it was necessary to devote special attention to the immunological relations between the two antigens and their antibodies. Serotype G is stable at 12° and transforms to E when the protozoa are transferred to 31° and cultivated at one fission/day, transformation being completed in 2–3 days.

METHODS

Organisms. Transformation was followed in *Paramecium aurelia*, variety 2, stock 28, originally isolated at Woods Hole, Massachusetts, U.S.A. Sera prepared against stock 3, isolated at Baltimore, Maryland, U.S.A., were also used. It should be noted that the variety 2 serotype G, considered here appears to be related to the serotype G of other varieties of *P. aurelia* (cf. Sonneborn, 1948; Beale, 1952). The variety 2 serotype E, however, is not related to the serotype E of variety 4 described by Sonneborn (1948).

Culture medium. The culture medium was: 0·15 % (w/v) Cerophyl infusion (Cerophyl Laboratories, Kansas City, Kansas, U.S.A.) with 1 g. $Na_2HPO_4.12H_2O$/l. The medium was inoculated with *Aerobacter cloacae* 1 or 2 days before use.

Antisera. Three antisera were used. Antigen was given intravenously to rabbits over a period of 3 weeks at two injections/week. Bleedings were made 1 week after the last injection. Serum P, no. 113, was made by injecting 5×10^6 homogenized protozoa of stock 3, serotype G, according to the method of Sonneborn (1950). This was used for immobilization tests. Serum P, no. 130, was made by injecting the cilia (see Preer & Preer, 1959) from 5×10^6 organisms of stock 3, serotype G. It was used for immobilization and precipitation tests, and gave only one zone of precipitation in gel diffusion tests against whole homogenates: that due to the serotype correlated antigen G. Serum P, no. 132, was obtained by injecting the cilia and trichocysts from 5×10^6 organisms of stock 28, serotype E. It was useful in both immobilization and gel diffusion tests. In gel diffusion tests, however, it was found to have a titre against certain heat stable non-immobilization antigens, as well as the heat-labile serotype correlated antigen. These heat stable antigens included the trichocyst antigen 1 (Preer & Preer, 1959). Bands due to these antigens were removed by absorbing the serum with an extract of boiled paramecia: 4×10^6 organisms of stock 3, serotype G, were suspended in 1 ml. saline and boiled for 10 min. Of the supernatant fluid, 0·143 ml. was added to 1 ml. of serum P, no. 132, to prepare the absorbed serum. It was then specific for immobilization antigen.

Antigens. Paramecia were filtered through sixteen layers of cheesecloth and concentrated by passing through a Massey-Harris automatic cream separator, modified as described by Preer & Preer (1959). A population count was carried out according to the method described by Sonneborn (1950). Further concentration was achieved by centrifuging for 2 min. in an International electric oil-testing centrifuge at 700 *g*. The supernatant fluid was discarded and the paramecia were resuspended in 0·45 M-KCl solution to a concentration of $1{\cdot}5 \times 10^6$ organisms/ml. and were then homogenized at 0° by using a special homogenizer with a tight-fitting Teflon tissue plunger. The homogenate was allowed to extract in a refrigerator for 24 hr. and was then centrifuged for 2 min. at 0° at 25,000 *g* in the high-speed head of an International refrigerated centrifuge. The supernatant fluid containing the soluble antigen was then stored in a deep-freeze until needed.

Gel diffusion tests. Assays were conducted by a technique of double diffusion in agar (Preer, 1956). About 0·01 ml. of serum was placed in the bottom of an agar-coated Pyrex tube of 3 mm. outside diameter; 0·6 % (w/v) agar in 0·9 % (w/v) saline with 0·01 % (w/v) thiomersalate at 60° was layered on to the serum to a depth of about 5 mm. and allowed to solidify. About 0·01 ml. of antigen was then layered on to the agar, and the tube capped with Picene cement. Tubes were placed at 25°, oriented horizontally, in order to minimize convection currents in the liquid layer (Preer & Telfer, 1957; Preer & Preer, 1959). At the region in the agar column where antigen diffusing from above met with specific antibodies diffusing from the serum layer below the agar, a band of precipitation was formed. Band positions were determined after one day with the aid of a low-power binocular microscope with a scale in one of the eyepieces. Positions were expressed as p, the distance from the antigen-agar interface to the centre of the 'band' (zone of precipitation) divided by the total agar length. Band position, p, is linearly related to the ratio of the concentration of the antigen to the concentration of the antibody (Preer, 1956). Thus, when antibody concentration is held constant, increases in the concentration of antigen result in displacement of the bands away from the antigen-agar interface. Hence a series of standard dilutions of the required antigen set against samples of a standard serum provided a reference curve for determining relative antigen concentrations. Absorption, or blocking tests, were performed by adding antigen to antiserum and then setting diffusion tubes with the mixture.

Experimental procedure. Cultures of stock 28, serotype G, were grown at 12° in 2 l. Erlenmeyer flasks and fed enough to allow no more than one fission every 10 days (Sonneborn, 1950), on the average. When a population of suitable size had been reached, the organisms were supplied with enough food to undergo one fission/day and transferred to 31° for transformation to proceed. At zero time (before transfer to 31°) and at frequent intervals during the transformation process, samples of the culture were removed and the immobilization titres for both serotypes G and E determined according to the methods described by Sonneborn (1950). Other organisms from the samples were extracted as described above in order to obtain antigens for gel diffusion tests. Every 24 hr. enough food was added to the cultures to maintain the rate of one fission/day. This process was continued for 4 days, long after transformation had been completed. Soluble antigen was then assayed by setting gel diffusion tests with suitable serum.

RESULTS

Antigens G and E and their cross-reactions

The immobilization titres (the greatest serum dilution giving complete and partial immobilization in 2 hr. at 27°) are given in Table 1, for the three sera and serotypes G and E of stock 28. It may be noted that, although each serum reacted best with its homologous serotype, cross-reactions were also observed, particularly in the case of the anti-E serum. Such cross-reactions have long been known (Sonneborn, 1947). Nevertheless, the cross-reactions observed here were somewhat greater than usually found between different serotypes of a single stock. It is unlikely in this case that the cross-reactions were due simply to the injection of a few organisms of the cross-reacting type in preparing the antiserum, since tests on samples of the paramecia used for injection contained none of the cross-reacting types. This evidence was especially convincing in the case of the anti-G sera, which were prepared against stock 3. Stock 3 has never been known to manifest serotype E; it probably lacks the gene for this type, just as do similar stocks studied by Finger (1957*b*).

In gel diffusion tests cross-reactions were even more marked. The anti-G serum P, no. 130, gave a zone of precipitation of almost equal intensity and position with both G and E antigens, and mixtures of the two antigens in varying ratios still yielded only one band. When the serum was absorbed with large quantities of the heterologous E antigen, no band was obtained with the E or G antigens. Absorption with a critical amount of E (near the equivalence ratio) produced a serum which did not give a band with the E antigen, but did with the homologous G antigen. However, such absorbed serum, while producing a zone of precipitation with the G and not E antigens and providing a qualitative test for G, proved useless in estimating the quantity of G antigen in the presence of E antigen. This was true because the addition of E antigen to extracts of G was found to displace the precipitation zone toward the serum-

Table 1. *The effect of several sera on serotypes G and E of stock* 28 *of* Paramecium aurelia

The highest dilution of serum giving complete immobilization and the highest dilution giving a visible effect in 2-hr. tests are given for each serotype and serum.

	Serotype G		Serotype E	
	Complete immobilization	Visible effect	Complete immobilization	Visible effect
Anti-G serum P, no. 113	1/25	1/400	—	1/50
Anti-G serum P, no. 130	1/25	1/800	—	1/25
Anti-E serum P, no. 132	1/12·5	1/100	1/100	1/1600

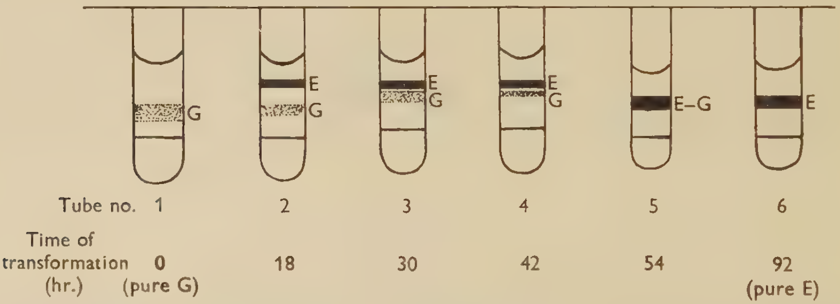

Fig. 1. Diagram of double diffusion tests on antigen extracts made from stock 28 paramecia at different intervals during transformation from serotype G to serotype E. All antigen extracts were set against anti-E serum P, no. 132. The serum, which normally contained antibodies against certain heat stable antigens as well as the heat-labile serotype correlated antigens, was made specific for the latter by absorption with an extract of boiled paramecia. Each tube was set against antigen extracted after the following periods of transformation: tube 1, 0 hr. (pure G); tube 2, 18 hr.; tube 3, 30 hr.; tube 4, 42 hr.; tube 5, 54 hr.; tube 6, 92 hr. (pure E). Serum P, no. 132, cross-reacted with antigen G, resulting in a fairly weak band (tube 1) which was clearly different from the band obtained with antigen E (tube 6). Two bands were formed while transformation was in progress (tubes 2, 3 and 4). The lower and weaker band was the one formed by antigen G; the upper and sharper band was formed by antigen E. For further details see text.

agar interface. Furthermore, the degree of displacement depended upon the quantity of E added. Since band position is the basis for the quantitative gel diffusion test, no means for measuring the quantity of G antigen in the presence of E was available with serum P, no. 130, as well as other anti-G sera.

The anti-E serum P, no. 132, also cross-reacted in gel diffusion tests, but less strongly than the G serum and in a somewhat different way. It gave a sharp band with the homologous E antigen and a weak band displaced toward the agar-serum interface with G antigen (see Fig. 1). When small quantities of G antigen were added to E antigen and set against serum P, no. 132, a single band was obtained with position and appearance just as if the E antigen were set unmixed. When larger quantities of G antigen were added to E, two bands appeared. The upper band toward the antigen-agar interface had the position and appearance of the E band, just as if no G antigen had been added, while the lower band (toward the serum-agar interface) was weak and appeared to be due to the G antigen. Addition of still larger concentrations of G antigen to the E gave no change in the upper E band, but progressively lowered the bottom band, showing clearly that it was due to the G antigen. It was thus evident that serum P, no. 132, could be used to determine the concentration of the E antigen, even in the mixtures of E and G—the E precipitation zone being recognized as a single, intense band, or as the upper band when two were formed. Since some of the antibody is precipitated by the G antigen in mixtures, one might expect an effect on the position of the E band. However, the proportion of the total antibody precipitated by the G antigen must be small, for no such displacement was noted. A standard curve relating the position of the E band to the concentration of the E antigen appears in Fig. 2. G antigen was present in most of the determinations and the ratio of G to E varied from 0:1 to 31:1. It can be seen that all the points fall on a

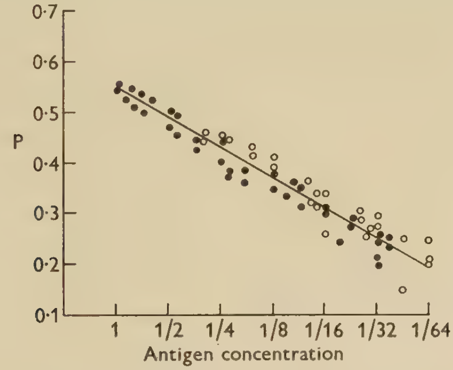

Fig. 2. Relation between band position p and antigen concentration for the E antigen set against anti-E serum P, no. 132. G extract was present in different amounts. For the points indicated with solid circles the E extract was in excess, the G:E ratio ranging from 0:1 to 1:2. For the points indicated with open circles the G antigen was in excess, the G:E ratio ranging from 2:1 to 31:1.

straight line. When the data were classified according to the G:E ratio, no effect of the G antigen on the position of the E band was apparent.

Gel diffusion tests on several other anti-G and anti-E sera showed that the sera P, no. 130, and P, no. 132, were typical—although the degree of cross-reactions differed somewhat. Anti-E sera showed much less cross-reaction than anti-G sera.

Transformation of serotype G to serotype E

Under the conditions employed, transformation from serotype G to serotype E was complete, every organism of type G transforming to E with exceedingly rare exceptions. In these cases, a few organisms (less than 1·0 %) could be found which reacted neither with anti-G nor anti-E sera in immobilization tests, and were evidently of a third unidentified serotype. Furthermore, transformation was synchronous, all organisms showing uniformly decreasing ability to react with anti-G and a simultaneous uniformly increasing ability to react with anti-E serum in immobilization tests. This behaviour is reflected by changes in the immobilization titre (Fig. 3). It can be seen that the immobilization titre for type G decreased, while the titre for type E showed a simultaneous increase. Thus during transformation both antigens were detectable at the same time, as other workers have reported for different transforming systems (see above). It may be noted that the maximum E titre was attained slightly before the G titre had reached its minimum value; but the difference was small and its significance is questionable.

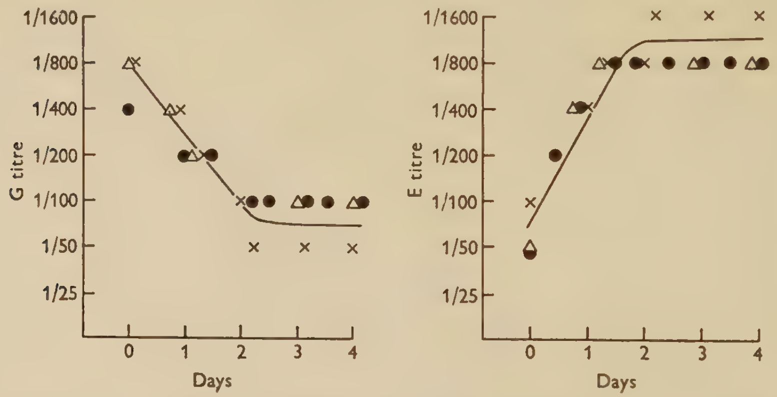

Fig. 3. Changes in immobilization titre during transformation of serotype G to serotype E in stock 28, variety 2, of *Paramecium aurelia*. The highest dilution of antiserum which partially immobilized organisms (gave definite avoiding reactions) is plotted against time of transformation. G organisms cross-reacted with the anti-E serum P, no. 132, and E organisms cross-reacted with the anti-G serum P, no. 113, when the sera were used in high concentration (see Table 1). Results from three independent experiments.

It was hoped that one could study quantitatively both the loss of the original antigen as well as the rise of the new one by using the gel diffusion technique. Unfortunately, it was possible to study quantitatively only the rise of the new antigen, since no sera were found which gave satisfactory resolution with antigen G. An attempt was made to estimate the different amounts of antigen G during the initial stages of transformation (i.e. where a distinct band was obtained with antigen G) by the use of serum P, no. 132 (see below), but consistent results were not obtained.

When antigen extracts from transforming organisms were tested against serum P, no. 132, two bands were clearly present during the initial stages of transformation (see Fig. 1). These were identified as the G and E bands of pure G and E organisms by absorption and mutual dilution procedures (Preer, 1956). There was no indication of the presence of molecules of intermediate specificity, for the appearance of the tubes could be duplicated by appropriate mixtures of pure G and E extracts. As transformation proceeded, the E band moved down toward the agar-serum interface, while the G band tended to move up. When transformation reached the point at which E was in excess of G, only one band was observed. It was possible, however, by using serum P, no. 130, which had been absorbed with just enough E antigen to prevent its reaction with E, to detect the presence of antigen G even after E became the major component of the transforming system. However, reliable quantitative estimates of the G antigen under these circumstances proved impossible, as noted above.

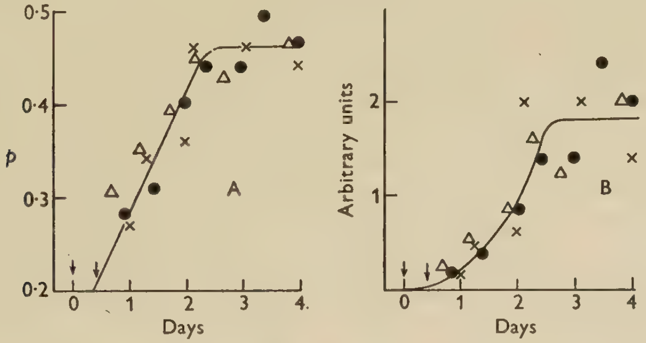

Fig. 4. Increase of antigen E during transformation of serotype G to serotype E in stock 28, variety 2, of *Paramecium aurelia*. A shows changes in the position (p) of the E band during transformation; B shows the amount of antigen in arbitrary units computed from p values in A. Each point represents an average obtained from five tubes set on the same antigen preparation. The variation between the highest and lowest value for p within each set of five tubes was generally less than 0·06. The highest variation observed (one case) was 0·14. The lines have been drawn by eye. The results are from three independent experiments. The arrows pointing downward at 0 and 10 hr. indicate that the quantity of antigen was below the indicated amount.

When the band positions p for antigen E were plotted against time of transformation, the graph obtained gave a logarithmic plot for the newly-synthesized antigen (Fig. 4A). It should be noted, as pointed out in the Methods section, that antigen was always extracted using the same concentration of organisms ($1{\cdot}5 \times 10^6$/ml.); hence the figures give a measure of the average amounts of antigen per Paramecium at each stage. When the amount of antigen was expressed in arbitrary units and these were plotted against time of transformation, a sigmoid curve was obtained (Fig. 4B).

If formation of new antigen begins at the time, or soon after the organisms are placed at 31°, it is clear that the curve (Fig. 4 B) cannot be linear. The results could only fit a linear curve if the assumption be made that formation of new antigen does not begin until after 15–18 hr. of cultivation at 31°. Even so the fit would be poor, and the hypothesis of logarithmic increase provides the best agreement with the data. It may be noted that Fig. 4A and the curve showing the changes in E titre (see Fig. 3) are practically identical.

DISCUSSION

The cross-reactions exhibited by serotypes G and E were somewhat greater than usually encountered in Paramecium, being particularly strong in the gel diffusion tests. The lack of sufficiently specific serum made it impossible to follow the original antigen quantitatively by gel diffusion as it disappeared during the transformation process; only the newly formed antigen was measured. Nevertheless, the G to E transformation was chosen for study because it is also somewhat unusual in that synchronous 100 % transformation may be readily obtained in mass culture. Furthermore, the gel diffusion technique made it possible to analyse the nature of the cross-reaction between the two antigens.

Cross-reactions of the type exhibited by the E antiserum are well known. The serum apparently contains a minor antibody fraction capable of reacting with both the G and E antigens, and a major fraction capable of reacting only with the E antigen. Thus an intense band was obtained with E extract and a weak band displaced toward the agar-serum interface with G extract. Mixtures of the two antigens gave one band as long as the E antigen was in excess, and two bands if the G antigen was in excess. Similar results were obtained by Oudin (1952) on hen and duck ovalbumin, and by Preer (1956) with artificially conjugated proteins.

We have not been able to find in the literature cross-reactions identical with those exhibited by the anti-G serum. The results may be explained by assuming that the serum contains one fraction of antibody capable of precipitating both G and E antigens, and another fraction capable of precipitating G antigen but capable of forming only soluble complexes with the heterologous E antigen. Since both antigens can react with all the antibody, mixtures of the two antigens in varying proportions can give but one band when diffused against the serum. In absorption experiments either antigen in sufficient quantity may react with all the antibody. It appears, however, that in absorption tests a critical amount of heterologous E antigen can remove the fraction of antibody capable of precipitating E antigen, but still leave antibody capable of forming soluble complex with E and precipitating G. When serum which has been absorbed to this degree is diffused against G antigen a band is formed. However, when it is set against E antigen, no band is formed. Mixtures of the two antigens set against the absorbed serum give a band (due to the G antigen) whose position is determined by both antigens (for both can react with the antibody). We were not able to provide decisive evidence in favour of this explanation, but Buchanan & Oudin (1957) reported that when duck ovalbumin was diffused against heterologous anti-hen ovalbumin, soluble complexes were formed which did not precipitate but did influence the position of the zone of precipitation in gel diffusion tests.

Sonneborn (1947 and private communication) considered two possibilities to account for cross-reactions. The first is that in each serotype there is one primary antigen present in high concentration, and that antigens corresponding to other serotypes may be present in low concentration as 'secondary' antigens. These secondary antigens induce low concentrations of antibody responsible for cross-reactions, but are not involved themselves to any significant degree in the immobilization reaction. The second possibility is that each serotype has only one immobilization antigen, but that because of close immunological relationship, antibody against the antigen of one serotype may react to some extent with the antigen of other serotypes. Our data show that antibody is formed which reacts with either the G or E antigens, even when obtained against type G of stock 3, which is apparently incapable of forming E antigen (see above). Such antibody would be expected on the basis of the second possibility. Furthermore, gel diffusion allows a direct test for secondary antigens in the extracts. The tests revealed none. Tests on mixtures of small amounts of E extract with G extract set with serum P, no. 132, showed the test was sufficiently sensitive to detect the presence of as little as one part of E antigen in 127 parts of G antigen. The basis for the cross-reaction between G and E in stock 28 is therefore, at least in part, due to structural similarity of the antigen molecules. No evidence for the presence of secondary antigens was obtained. This is in agreement with the conclusions reached by other workers (see Beale, 1954; Finger, 1957*a*). Finger (1957*a*) suggested that the serological basis for the cross-reactions between immobilization antigens controlled by different alleles of the same genetic locus lies in similarity of structure between the antigens. The results presented here would justify extending this conclusion to apply to all cases of cross-reacting antigens in Paramecium, whether controlled by the same or different loci.

There is no critical evidence at the moment as to whether, in transformation, the old antigen contributes to the new antigen or not. Sonneborn & Whallon (1950) reported that in transformations from type A to type B in stock 51, variety 4, the original A titre showed very little detectable decrease until the B titre had risen to its final value. Our data indicate that the immobilization titres can be used, within limits, as a valid indication of the presence of old and new antigen. It is therefore likely that in the A to B transformation the synthesis of the new antigen is not necessarily dependent upon the loss of the old one, and hence the new antigen is not formed from the old by a change in the configuration of the molecule. It should also be pointed out that it is very difficult to conceive of the role of the genes in the determination of specificity if transformation involves a change from one specific antigen into another, rather than a *de novo* synthesis of new antigen, for the specific nature of each new serotype formed is controlled by only one specific locus, and is completely independent of genes determining the specific nature of the old serotype in which it arises. The shape of the curve for the synthesis of the new antigen makes more sense within the context of a hypothesis assuming *de novo* synthesis than one invoking a change in the configuration of a pre-existing molecule. Reliable data on this most important aspect of the problem, the loss of the original antigen, are unfortunately scarce. Kinetic studies of the transformation in more suitable systems using the techniques described in this paper might throw light on the matter, particularly when combined with the use of radioactive isotopes.

Gel diffusion tests on extracts of transforming paramecia demonstrated the presence of both the old and new antigens. The results of such tests could be duplicated by mixing extracts of untransformed and fully transformed organisms in varying proportions. There is no evidence for the formation of molecules of intermediate specificity. The autocatalytic kinetics of increase shown by the newly formed antigen during transformation is similar to the kinetics of adaptive enzyme formation (see Spiegelman, 1956). Indeed the capacity for induction of a new complex molecule at will in induced serotype transformation is reminiscent of the induction of adaptive enzymes. Possibly some of the techniques which have been used in studying adaptive enzyme formation might be useful in studying the mechanism of transformation in Paramecium, now that quantitative methods for measuring the antigens *in vitro* are available.

In a recent review Beale (1957) pointed out the difficulties standing in the way of a simple hypothesis to explain serotype determination in *Paramecium aurelia*. Recent evidence indicates that the serotype-correlated antigens have the properties of a protein. This, together with the similarities between antigenic transformation and adaptive enzyme formation, suggest that the explanation for serotypes and serotype determination and transformation is closely tied to the problem of protein synthesis. Various possibilities have been summarized elsewhere (Preer, 1958).

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Synthesis of Flagella by Amino Acid-Requiring Mutants of *Salmonella typhimurium*

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SUMMARY: Amino acid-requiring mutants of *Salmonella typhimurium* were examined for their ability to regenerate flagella when incubated with a source of nitrogen and of energy, but in the absence of the amino acid required for growth. The mutants fell into two major groups and one intermediate group. The major groups were: (*a*) mutants requiring for growth an amino acid (such as leucine or tyrosine) present in flagellin and unable to regenerate flagella in the absence of this amino acid. (*b*) mutants requiring for growth an amino acid (such as tryptophan or cysteine) not present in flagellin and able to regenerate flagella in the absence of this amino acid. The intermediate group (*c*) contained mutants requiring for growth an amino acid (such as histidine or methionine) present in small amount in flagellin and able to regenerate flagella in the absence of this amino acid. With all the mutants tested there was no significant net synthesis of RNA or protein in the absence of the amino acid required for growth, but DNA synthesis did occur.

Bacterial flagella, unlike those of protozoa and algae, consist only of protein. Weibull (1953) and Rinker, Kobayashi & Koffler (1957) showed that flagellar protein (flagellin) from *Proteus vulgaris* was homogeneous, had an approximate molecular weight of 17,000, possessed an *N*-terminal alanine and was unusual in containing no tryptophan, histidine, proline or hydroxyproline. It differs from flagellin of *Salmonella typhimurium* which lacks tryptophan, cysteine and hydroxyproline (Mr R. P. Ambler and Mr M. W. Rees, Department of Biochemistry, Cambridge, personal communication). The M protein of group A haemolytic streptococci is similar to flagellin in being an incomplete protein, for it contains little or no aromatic amino acids. It is possible to remove the M protein from the streptococci by trypsin digestion; the trypsin-treated organisms require amino acids, polypeptides and glucose for the synthesis of the M protein; tryptophan and phenylalanine are essential for growth but not for synthesis of M protein in a chemically-defined medium (Fox & Krampitz, 1956).

Stocker (1957) showed that flagella could be removed mechanically from *Salmonella typhimurium* without affecting the viability of the organisms and that in a nutrient broth these organisms were capable of regenerating their flagella. As bacterial flagella consist only of protein, regenerating flagella appeared to be a suitable system for studying by morphological and functional tests the factors which affect the synthesis of a single protein. In the present work the ability of amino acid-requiring mutants of *Salmonella typhimurium* to regenerate flagella in the presence and absence of their amino acid growth requirements was investigated and compared with other synthetic abilities under the same conditions.

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METHODS

Organisms and growth media

The mutant strains of *Salmonella typhimurium* used are detailed in Table 1. The cultures were maintained on Dorset egg slopes which were kept at room temperature.

For the preparation of washed suspensions, organisms were grown in nutrient broth made from a tryptic digest of horse meat; 50 ml. broth was inoculated with 1 ml. of an overnight culture and incubated aerobically without shaking for 4 hr. at 37°; the organisms were at the end of the logarithmic phase of growth after this period. The organisms were harvested by centrifugation, washed twice with 0·01 M-phosphate buffer (pH 7·0) and resuspended in the same buffer at a concentration equiv. 10 mg. dry wt./ml. Starved preparations of the amino acid-requiring mutants were obtained by resuspending the washed organisms at a concentration equiv. to 1 mg. dry wt./ml. in a minimal medium consisting of (%, w/v) K_2HPO_4, 0·7; KH_2PO_4, 0·2; $Na_3(citrate).5H_2O$, 0·05; $MgSO_4.7H_2O$, 0·01; $(NH_4)_2SO_4$, 0·1; glucose, 0·2. After aerobic incubation for 30 min. at 37° the organisms were harvested by centrifugation, washed and resuspended in buffer as before.

Table 1. *Strains of* Salmonella typhimurium *used which require certain amino acids for growth*

Strain no.	Parent strain	Growth requirement	Lab. ref. no.	Source
LT2	—	—	LT2	Zinder & Lederberg (1952)
462	533	Tyrosine	SL185	Dr M. H. Plough
48OM	533	Leucine	SL186	Dr M. H. Plough
meA-6	LT2	Methionine	SL262	Demerec *et al.* (1956)
tryA-8	LT2	Tryptophan	SL282	
hiA-6	LT2	Histidine	SL286	
hiB-20	LT2	Histidine	SL287	
hiC-15	LT2	Histidine	SL288	
hiD-10	LT2	Histidine	SL289	
cys 1	LT2	Cysteine	SL291	
glu 1	LT2	Glutamic acid	SL443	

The dry weights of the organisms in suspension were determined from optical density measurements at 420 mμ by using a Unicam SP 500 spectrophotometer and a calibration curve for the organisms prepared by drying samples of known optical density to constant weight at 105°.

The flagella were detached mechanically from the bacteria. Ten ml. of the bacterial suspension in buffer was placed in a 25 ml. masticator (MSE 77313) and blended at room temperature by a MSE microhomogenizer. One to 2 min. blending at 12,000 rev./min. was sufficient to decrease the number of flagellated bacteria to $< 1\%$ of the total.

Regeneration of bacterial flagella

Regeneration of flagella was carried out in 250 ml. conical flasks containing phosphate buffer (pH 7·0; final concentration 0·01 M), glucose 0·2 % (w/v) and the following amino acids each in a concentration of 0·1 mg./ml.: glycine, L-glutamic acid, L-aspartic acid, L-valine, L-leucine, L-isoleucine, L-arginine, L-proline, L-alanine, L-histidine, L-lysine, L-tyrosine, L-cysteine, DL-threonine, DL- ornithine, DL-tryptophan and DL-phenylalanine. Organisms were added to a final concentration equiv. to 0·2 mg. dry wt./ml.; 100 ml. medium was incubated at 37°, in shaken flasks to improve aeration.

Bacterial growth was estimated from the optical density of the suspension by using a calibration curve as described above.

Flagellar regeneration was determined as follows, (*a*) Motility determinations on 0·2 ml. samples added to 1·8 ml. nutrient broth containing 10 μg. chloramphenicol/ml. At this concentration chloramphenicol prevented the synthesis of flagella but had no effect on the motility of the organisms. The % organisms with translational motility was determined microscopically. (*b*) Flagellar staining. The bacterial flagella were stained by Leifson's (1951) method as modified by Quadling (1958). The mean number of flagella/bacterium and the % flagellated bacteria were determined.

Nucleic acid and protein synthesis. The fractionation procedure was based on that of Roberts, Cowie, Abelson, Bolton & Britten (1955). The organisms in 10 ml. samples were harvested by centrifugation, resuspended in 3 ml. 5 % (w/v) trichloroacetic acid (TCA) and held at 4° for 2 hr. The precipitate was harvested by centrifugation and after washing once with 5 % (w/v) TCA was extracted for 30 min. at 45° in 3 ml. 75 % (v/v) ethanol in water. The suspension was centrifuged, the precipitate extracted for 30 min. at 45° in 2 ml. 75 % (v/v) ethanol in water + 2 ml. ether. The ethanol and ethanol + ether extracts were combined and after the addition of 5 ml. water were extracted three times with 5 ml. ether to remove the lipid fraction, which was discarded. The aqueous layer containing the ethanol-soluble protein was taken to dryness on a boiling water bath; the ethanol-soluble protein was then redissolved in 0·5 ml. N-NaOH and estimated colorimetrically with Folin's reagent by the method of Lowry, Roseborough, Farr & Randall (1951). The nucleic acid was extracted from the residue of the ethanol + ether extract with perchloric acid; two 20 min. extractions at 70° with 2 ml. 0·5 N-perchloric acid were sufficient to remove all the nucleic acid. The supernatant fluids from these two extractions were combined and made up to 5 ml. with 0·5 N-perchloric acid. In certain experiments the nucleic acid was separated into the RNA and DNA fractions by the method of Ogur & Rosen (1950). Total nucleic acid was determined after suitable dilution by estimating the optical density at 260 mμ with a Unicam SP 500 spectrophotometer. DNA was determined by the diphenylamine method of Burton (1956) and RNA by the orcinol method of Morse & Carter (1949). The residue after the extraction of nucleic acid was taken up in 5·0 ml. N-NaOH and the protein content estimated colorimetrically with Folin's reagent (Lowry *et al.* 1951).

Induction and estimation of nitratase activity in washed suspensions of Salmonella typhimurium. The induction was carried out anaerobically in Thunberg tubes at 37° in a system consisting of: 0·05 M-phosphate buffer (pH 7·0); 0·01 M-KNO_3; amino acid mixture as for the regeneration of flagella; 2·0 % (w/v) glucose; organisms corresponding to a final concentration equiv. to 0·4 mg. dry wt./ml. After incubation for 2 hr. the organisms were harvested by centrifugation, washed once with distilled water, suspended in distilled water, and the concentration determined turbidimetrically. The nitratase activity was determined by the method of Pollock (1946).

RESULTS

Conditions for flagellar regeneration

Both the degree of flagellation and the ability of suspensions of *Salmonella typhimurium* to regenerate their flagella after mechanical removal were found to depend on the conditions under which the organisms were grown. Flagellin can account for up to 1 % of the dry weight of the organisms grown in nutrient broth when there are 4–5 flagella/bacterium. Organisms grown in the minimal medium, however, have fewer flagella/bacterium, and after mechanical removal their flagella do not regenerate as well as those of organisms grown in nutrient broth. Although it would have been more satisfactory to use organisms grown in a chemically defined medium, in view of their relative inability to regenerate their flagella, *S. typhimurium* grown in a nutrient broth was used.

The ability of different nitrogen sources to promote flagellar regeneration by the nutritionally non-exacting strain LT 2 of *Salmonella typhimurium* in a chemically defined medium was studied. The nitrogen sources included ammonium salts, single amino acids and a complete mixture of amino acids. Only the complete mixture of amino acids gave consistently reproducible results as good as those obtained with organisms incubated in nutrient broth after the mechanical removal of their flagella. The addition of a purine and pyrimidine mixture (xanthine, hypoxanthine, adenine, guanine, thymine, uracil) in a concentration of 10 μg. each base/ml. had little or no effect on regeneration and was therefore not used routinely. Bacterial growth and the regeneration of flagella by the nutritionally non-exacting strain LT 2 of *S. typhimurium* in the chemically defined media are shown in Table 2. Regeneration occurred rapidly in this medium and after 1 hr. nearly all the organisms had regained their motility. The difference between the % motile organisms (20–30 %) and % flagellated organisms (90–93 %) after 30 min. incubation was due to the fact that for the determination of % flagellated bacteria, every bacterium possessing one or more flagella (irrespective of length) was scored as flagellated, whereas a flagellum has to be a certain minimum length before the organisms become motile. Organisms in buffer alone showed no change in optical density and were unable to regenerate their flagella. The average number of flagella/bacterium after regeneration was always less than in the original culture. Concomitantly with the regeneration of flagella by strain

LT 2 the synthesis of nucleic acid and protein was estimated. The results of a typical experiment are shown in Table 3, in which the values for the ethanol-soluble protein and the residual protein are combined. Growth occurred, in all cases except in phosphate buffer, and there was a good correlation between the ability to regenerate flagella and to synthesize nucleic acid and protein.

Table 2. *The effect of purines and pyrimidines on growth and flagellar regeneration of* Salmonella typhimurium *strain LT* 2

	Regeneration medium		Regeneration medium + purines + pyrimidines		Control (phosphate buffer)	
Time (min.)	Suspension density (equiv. mg. dry wt./ml.)	Motility* %	Suspension density (equiv. mg. dry wt./ml.)	Motility* %	Suspension density (equiv. mg. dry wt./ml.)	Motility* %
0	0·2	1 (< 1)	0·2	<1 (< 1)	0·2	< 1 (< 1)
30	0·24	30 (93)	0·26	30 (90)	0·2	< 1 (< 1)
60	0·30	80 (96)	0·32	80 (98)	0·2	< 1 (< 1)
90	0·32	90 (96)	0·36	90 (90)	0·2	< 1 (< 1)
120	0·34	90 (94)	0·38	90 (98)	0·19	< 1 (< 1)

* The figures in parentheses are % of flagellated bacteria determined in stained preparations.

Table 3. *The effect of purines and pyrimidines on nucleic acid and protein synthesis by* Salmonella typhimurium *strain LT* 2

	Regeneration medium			Regeneration medium + purines + pyrimidines			Control (phosphate buffer)		
Time (min.)	RNA*	DNA*	Protein†	RNA*	DNA*	Protein†	RNA*	DNA*	Protein†
0	347	74	149	347	74	149	347	74	149
30	388	87	168	425	99	176	338	74	151
60	462	105	197	485	115	223	—	77	157
90	513	128	242	567	130	264	342	70	159
120	530	155	255	542	150	278	368	63	165

* μg. nucleic acid/10 ml. sample.
† μg. protein-N/10 ml. sample.

Flagellar synthesis by amino acid-requiring mutants of Salmonella typhimurium

The amino acid-requiring mutants examined for their ability to regenerate flagella can be divided into two major groups *a* and *b* and one intermediate group *c*.

(*a*) *Mutants requiring an amino acid present in flagellin; unable to regenerate flagella in the absence of the amino acid required for growth*

Glutamic acid, leucine and tyrosine are all present in the flagellin of *Salmonella typhimurium* and one strain exacting for each of these amino acids was tested. All strains grew and synthesized flagella in the complete regeneration medium (Table 4), although the recovery of motility after incubation for 2 hr. was not as good as in the nutritionally non-exacting strain LT 2. When the amino acid required for growth was omitted from the medium there was

Table 4. *The effect of essential amino acid nutrients on growth and flagellar regeneration by amino acid-requiring strains of* Salmonella typhimurium

			Regeneration medium		Regeneration medium lacking growth requirement		Control (phosphate buffer + glucose)	
Strain	Growth requirement	Starvation	Suspension optical density % increase	Motility* (%)	Suspension optical density % increase	Motility* (%)	Suspension optical density % increase	Motility* (%)
Group a								
SL185	Leucine	.	35	40 (50)	7	< 1 (1)	0	< 1
SL185	Leucine	+	65	10 (60)	13	< 1 (<1)	− 1	< 1
SL186	Tyrosine	.	78	50 (40)	− 3	< 1 (<1)	0	< 1
SL443	Glutamic acid	.	90	20	−13	< 1	−15	< 1
Group b								
SL291	Cysteine	.	60	40 (39)	16	50 (35)	− 7	< 1
SL282	Tryptophan	.	45	70 (90)	2	65 (85)	−12	< 1
SL282	Tryptophan	+	78	80	− 1	50	− 1	< 1
Group c								
SL286	Histidine	.	66	70	− 1	30	−17	10
SL286	Histidine	+	106	80	−12	10	−13	2
SL287	Histidine	.	100	70	6	40	−15	5
SL287	Histidine	+	120	70	− 6	40	2	2
SL288	Histidine	.	110	80	6	40	− 6	10
SL288	Histidine	+	110	70	6	40	− 8	5
SL299	Histidine	.	55	70	3	60	− 9	< 1
SL299	Histidine	+	92	80	− 6	40	− 6	10
SL262	Methionine	.	76	60	13	10	−10	< 1

* The figures in parentheses are % flagellated bacteria in stained preparations.

Table 5. *The effect of essential nutrient amino acids on nucleic acid and protein synthesis by amino acid-requiring strains of* Salmonella typhimurium

			% increase after aerobic incubation for 2 hr. at 37°								
			Regeneration medium			Regeneration medium lacking growth requirement			Control (phosphate buffer + glucose)		
Strain	Growth requirement	Starvation	RNA	DNA	Protein	RNA	DNA	Protein	RNA	DNA	Protein
roup a											
SL185	Leucine	.	40	150	47	− 2	100	2	17	− 7	−1
SL185	Leucine	+	10	55	38	− 8	17	− 3	− 7	8	5
SL186	Tyrosine	.	38	108	102	− 1	77	− 2	−21	10	3
SL443	Glutamic acid	.	63	114	98	−10	26	−12	−18	23	0
roup b											
SL291	Cysteine	.	42	100	61	− 5	60	3	−14	0	−6
SL282	Tryptophan	.	25	113	54	0	107	4	0	3	6
SL282	Tryptophan	+	60	170	110	− 3	43	9	−10	45	2
roup c											
SL262	Methionine	.	30	94	40	−16	33	−10	−23	− 4	−4
SL286	Histidine	.	13	53	23	6	40	1	4	− 4	−4

little change in the optical density of the corresponding nutritionally exacting mutants and the organisms did not recover their motility; i.e. they were unable to regenerate their flagella (Table 4). The most noticeable feature is that although net RNA and protein synthesis did not occur (the small changes are within the limits of the experimental error) in the presence of a source of energy and of nitrogen but in the absence of the specific amino acid required for growth, the organisms were still capable of synthesizing DNA (Table 5). The results are similar to those obtained with *Escherichia coli* mutants by Pardee & Prestridge (1956).

(*b*) *Mutants requiring for growth an amino acid absent from flagellin and able to regenerate flagella in the absence of their growth requirement.*

The amino acids tryptophan, cysteine and hydroxyproline are not present in the flagellin of *Salmonella typhimurium*. Growth and flagellar regeneration were followed in a cysteine-requiring mutant (SL 291) and in a tryptophan-requiring mutant (SL 282) of *S. typhimurium* (Table 4). There was very good regeneration of flagella by the tryptophan-requiring mutant in the presence and in the absence of tryptophan. Previous starvation of the organisms had little effect on regeneration. There was little or no change in the optical density of a suspension of strain SL 282 during incubation for 2 hr. in medium which lacked tryptophan. The cysteine-requiring mutant behaved similarly except that its recovery of motility even in the presence of cysteine was poorer than that of the tryptophan-requiring mutant (Table 4). Although there was a 16 % increase in the optical density of a suspension of strain SL 291 in a medium lacking cysteine, there was no accompanying change in the protein content (Table 5). Both the tryptophan- and cysteine-requiring mutants behaved like the other mutants in respect of nucleic acid and protein synthesis in the regeneration medium, with or without their amino acid growth requirement (Table 5). The addition of tryptophan to a suspension of strain SL 282 in regeneration medium lacking tryptophan resulted in the immediate synthesis of RNA and protein; synthesis of DNA continued at the same rate as before (Fig. 1).

As a further check on the results about nucleic acid synthesis by the amino acid-requiring mutants of *Salmonella typhimurium* the nucleic acid was fractionated into the RNA and DNA components at the end of a regeneration test of strain SL 282 (tryptophan-requiring). The results (Table 6) are consistent with those obtained in the earlier experiments. There was little or no RNA synthesis in the absence of the amino acid required for growth, but there was considerable synthesis of DNA.

(*c*) *Mutants which require for growth an amino acid present in flagellin, but which are able to regenerate flagella in the absence of this amino acid*

The mutants examined in this group were histidine- and methionine-requiring strains of *Salmonella typhimurium*. A fairly high proportion of organisms in a deflagellated suspension regenerated their flagella in a medium which lacked the amino acid required for growth (Table 4) although the amino acids required for growth are present in flagellin. The histidine-requiring strains were typical of these organisms. Strain SL 286 was first examined; because of the rather unexpected results obtained mutants blocked at other histidine loci were also tested (Tables 1 and 4). All the histidine-requiring strains of *S. typhimurium* regenerated their flagella readily in complete medium; at least 70% of the organisms were motile after incubation for 2 hr. Starvation of the suspensions in a chemically defined medium which lacked histidine, before regeneration in complete medium, had no effect on the regeneration of flagella. In medium lacking histidine there was a moderately good recovery of motility, varying from 30 to 60 % in the non-starved suspension

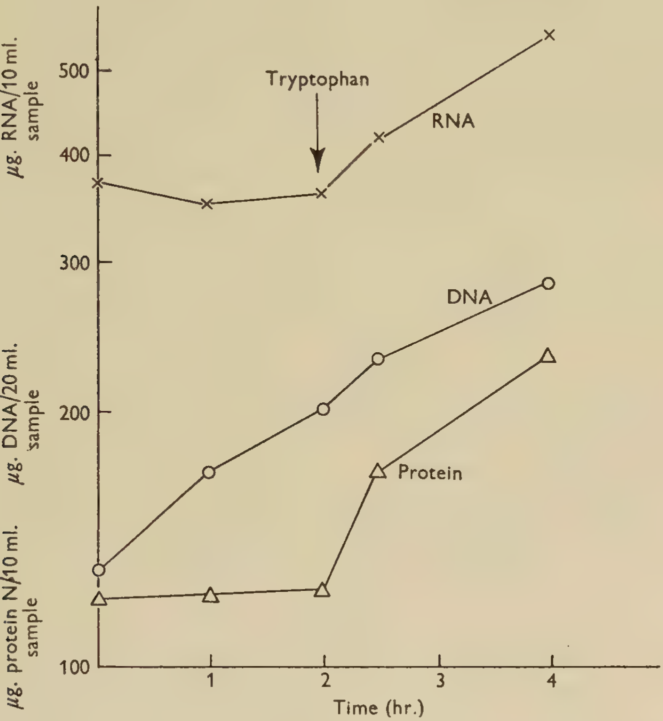

Fig. 1. The effect of the addition of tryptophan on nucleic acid and protein synthesis by *Salmonella typhimurium*, strain SL 282 (tryptophan-requiring).

Table 6. *The effect of tryptophan on the synthesis of RNA and DNA by the tryptophan-requiring strain SL* 282 *of* Salmonella typhimurium

	% increase after aerobic incubation for 2 hr. at 37°					
	Regeneration medium		Regeneration medium lacking tryptophan		Control (phosphate buffer + glucose)	
Method of estimation	RNA	DNA	RNA	DNA	RNA	DNA
Diphenylamine	—	110	—	53	—	8
Log I_0/I at 260 mμ	60	120	−2	51	−10	5

and from 10 to 40 % in the starved suspension, although the optical density changed little during the 2 hr. period. When the histidine-requiring strains were incubated in the absence of a nitrogen source there was a small recovery of motility but in no case did this exceed the recovery by organisms incubated in medium lacking histidine. The methionine-requiring mutant of *S. typhimurium* (SL 262) behaved like the histidine-requiring strains, although the recovery of motility in the absence of the amino acid required for growth was not good. The synthesis of nucleic acid and protein by strain SL 262 (methionine-requiring) and strain SL 286 (histidine-requiring) was similar to that by the other amino acid-requiring strains of *S. typhimurium*.

Nitratase synthesis by amino acid-requiring mutants of Salmonella typhimurium

The ability of amino acid-requiring mutants of *Salmonella typhimurium* to synthesize protein in the absence of their growth requirement was examined by studying the synthesis of the adaptive (inducible) nitratase system in strain SL 282 (tryptophan-requiring) and strain SL 443 (glutamic acid-requiring). The increase in nitratase activity was inhibited by growth-inhibitory concentrations of chloramphenicol; this presumably resulted from an inhibition of protein synthesis. When organisms of either strain were incubated in the complete adaptation medium there was an approximate five-fold increase in the nitratase activity during the 2 hr. anaerobic incubation. When, however, the amino acid required for growth was omitted from the amino acid mixture there was little or no change in the nitratase activity of the suspensions. Thus, in the absence of the amino acid required for growth the two auxotrophic mutants did not synthesize nitratase (Table 7).

Table 7. *The effect of various substances on the synthesis of nitratase by amino acid-requiring strains of* Salmonella typhimurium

	Additions to the basal adaptation medium					Nitratase activity (μmole NO_2/mg./hr.)	
Strain	Glutamic acid (0·1 mg./ml.)	Tryptophan (0·1 mg./ml.)	Purine and pyrimidine mixture (10 μg. of each base/ml.)	Chloramphenicol (10 μg./ml.)	KNO_3 (0·01 M)	Initial	Final
SL 282. Tryptophan-requiring	+	+	+	−	−	1·75	1·69
	+	+	+	+	+	1·75	2·12
	+	+	+	−	+	1·75	8·74
	+	+	−	−	+	1·75	8·76
	+	−	+	−	+	1·75	1·64
	+	−	−	−	+	1·75	1·72
SL 443. Glutamic acid requiring	+	+	+	−	−	1·33	0·91
	+	+	+	+	+	1·33	1·33
	+	+	+	−	+	1·33	6·5
	+	+	−	−	+	1·33	4·6
	−	+	+	−	+	1·33	1·02
	−	+	−	−	+	1·33	1·43

DISCUSSION

Adaptive enzyme formation by washed suspensions of bacteria was first used to study the formation of individual proteins; later Monod and his collaborators studied the differential synthesis of enzymes in logarithmically growing cultures of *Escherichia coli* (see Cohn, 1957). In both these approaches, although the synthesis of only one protein is followed, other proteins are also being synthesized. It was with the idea of overcoming this limitation that the study of the synthesis of flagella by amino acid-requiring mutants of *Salmonella typhimurium* was begun. Flagella are ideal as a single protein for synthetic studies because they consist only of protein and constitute up to 1% of the dry weight of the organisms. This protein is well characterized serologically and chemically and is easily isolated and purified. A further advantage of this system is the possibility of studying the behaviour of individual bacteria within the population. The system has a major disadvantage in that although qualitative estimation of flagellar synthesis is easy, quantitative estimation is difficult.

When the amino acid-requiring mutants were incubated in the regeneration medium from which the amino acid required for growth had been omitted, there was little or no net synthesis of RNA or protein, but DNA was synthesized. The results are similar to those of Pardee & Prestridge (1956) who used auxotrophic mutants of *Escherichia coli.* Although increases in RNA and protein were not detected when the salmonella strains were incubated in the absence of their growth requirement, certain strains regenerated their flagella under these conditions. The behaviour of the glutamic acid-, leucine- and tyrosine-requiring strains (group a) and the tryptophan- and cysteine-requiring strains (group b) is to be expected since it is plausible to assume that in the absence of their amino acid growth requirement, strains which require an amino acid for growth would synthesize proteins which lacked that amino acid but not proteins which contained it. The behaviour of the histidine-requiring mutants and the methionine-requiring mutant (group c) is difficult to reconcile with that of the mutants in group a. Histidine and methionine occur to a limited extent in flagellin (approximately 3 mole/10^5 g. compared to 78 mole/10^5 g. for leucine and 21 mole/10^5 g. for tyrosine) and although the synthesis of flagellin might be accounted for by the presence of these amino acids in an amino acid pool, the ability of starved preparations to regenerate flagella in the absence of their amino acid growth requirement makes this unlikely. The demonstration of protein turnover in resting suspensions of *E. coli* (Mandelstam, 1958) suggests an explanation. If one assumes a similar turnover in *Salmonella typhimurium* then a limited amount of histidine and methionine would be available for the synthesis of protein and the relative amount of a particular protein synthesized would depend on the number of histidine or methionine residues/ protein molecule. Flagellin would be at an advantage in that it contains only one histidine and one methionine residue/ flagellin molecule of mol. wt. 30,000. Consequently the synthesis of flagellin would be more probable than that of other proteins containing more histidine or methionine residues/protein molecule. The failure to show synthesis of RNA associated with the regeneration of flagella by the amino acid-requiring mutants when incubated in the absence of the amino acid required for growth was no doubt due to the insensitivity of the estimation procedures used. Any increase in RNA occurring during the synthesis of flagella by amino acid-requiring mutants in the absence of their growth requirement would be less than 1 %; this increase would not be detected by the methods used to estimate RNA.

The ability to regenerate flagella is not constant among the individuals of a bacterial population. Some organisms do not regenerate flagella during the period of the test and the proportion of these varies from strain to strain. Different organisms are probably in different phases of division when harvested and the synthesis of flagella might occur only during particular periods of this cycle. This explanation would be in accord with the results of Lark & Maaløe (1956) on the synthesis of nucleic acid and protein by synchronously dividing cultures of *Salmonella typhimurium*.

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Inhibition of Growth of *Tetrahymena piriformis* by Certain Steroids

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SUMMARY: The growth of *Tetrahymena piriformis W* is inhibited by several steroids of mammalian origin. The degree of inhibition is related to the molecular configuration. All of the effective growth inhibitors were of the carbon-21 or pregnane series. Hydroxyl substitution at carbon-11 enhanced inhibition, while hydroxyl substitution at carbon-17 lowered the potency of inhibition. The inhibition of growth induced by these steroids was antagonized by stigmasterol or cholesterol. The mode of action of these compounds is discussed with regard to possible competitive inhibition with some metabolite essential for this organism.

The growth of *Tetrahymena piriformis W* has been reported to be inhibited by a number of compounds which are antagonized by stigmasterol or cholesterol (Conner, 1957; Conner & Nakatani, 1958; Dewey, Heinrich & Kidder, 1958). An understanding of how the inhibition of such unrelated compounds as 2:4-dinitrophenol (Conner, 1957), colchicine, deoxycorticosterone (Conner, & Nakatani 1958) and 6-methyl purine (Dewey *et al.* 1958) is antagonized by certain sterols has not yet been reached. It was noticed, however, that the various steroids differed in their ability to inhibit the growth of this organism, and the greater the degree of inhibition the higher the concentration of stigmasterol required to annul this effect. This led to a study of the molecular configuration of different steroids with regard to potency of biological action.

METHODS

Tetrahymena piriformis W was grown in a modified chemically defined medium by techniques based on those developed by Kidder *et al.* (1951) and Dewey *et al.* (1951). The composition of the medium is given in Table 1.

The steroids were suspended in warm Tween 80 by the aid of a motor-driven Teflon homogenizer in preparation for testing. These solutions were sterilized separately and added aseptically while still warm. All the steroids tested were soluble to *c.* 10^{-3}M in the culture medium used. The following steroids were tested at final concentrations of 1, 2·5, 5, 7·5 and 10×10^{-4}M: two preparations of deoxycorticosterone, two samples of progesterone, two samples of cortisone, one of 11-dehydrocorticosterone, corticosterone, 17-hydroxydeoxycorticosterone, 9-α-flurocortisone, 17-hydroxycorticosterone, 1-dehydrocortisone, 1-dehydro-17-hydroxycorticosterone, diethylstilbesterol, testosterone, estradiol (Merck, Sharp and Dohne, Inc., given by Dr L. Sarret, Rahway, New Jersey, U.S.A.).

The sterols stigmasterol (from Dr O. Behrens, Eli Lilly Co., Indianapolis,

Table 1. *Composition of medium used for the growth of* Tetrahymena piriformis W.

Compound	(μg./ml.)	Compound	(μg./ml.)
DL-Alanine	110·0	$MgSO_4.7H_2O$	100·0
L-Arginine	206·0	$Fe(NH_4)_2(SO_4)_2.6H_2O$	25·0
L-Aspartic acid	122·0	$MnCl_2.4H_2O$	0·50
Glycine	10·0	$ZnCl_2$	0·05
L-Glutamic acid	233·0	$CaCl_2.2H_2O$	50·00
L-Histidine	87·0	$CuCl_2.2H_2O$	5·00
DL-Isoleucine	276·0	$FeCl_3.6H_2O$	1·25
L-Leucine	344·0	K_2HPO_4	1000·0
L-Lysine	272·0	KH_2PO_4	1000·0
DL-Methionine	248·0		
L-Phenylalanine	160·0	Guanylic acid	30·0
L-Proline	250·0	Adenylic acid	20·0
DL-Serine	394·0	Uracil	10·0
DL-Threonine	326·0	Cytidylic acid	20·0
L-Tryptophan	72·0		
DL-Valine	162·0	Glucose	2500·0
Calcium pantothenate	0·10	Sodium acetate	1000·0
Nicotinamide	0·10		
Pyridoxine hydrochloride	1·0	Tween 80	10,000·0
Pyridoxal hydrochloride	0·10		
Pyridoxamine hydrochloride	0·10	DL-Thioctic acid	0·002
Riboflavin	0·10		
Pteroylglutamic acid	0·01	Disodium ethylenediamine tetraacetic acid (EDTA)	20·0
Thiamine hydrochloride	1·00		
Biotin (free acid)	0·0005		
Choline chloride	1·00		

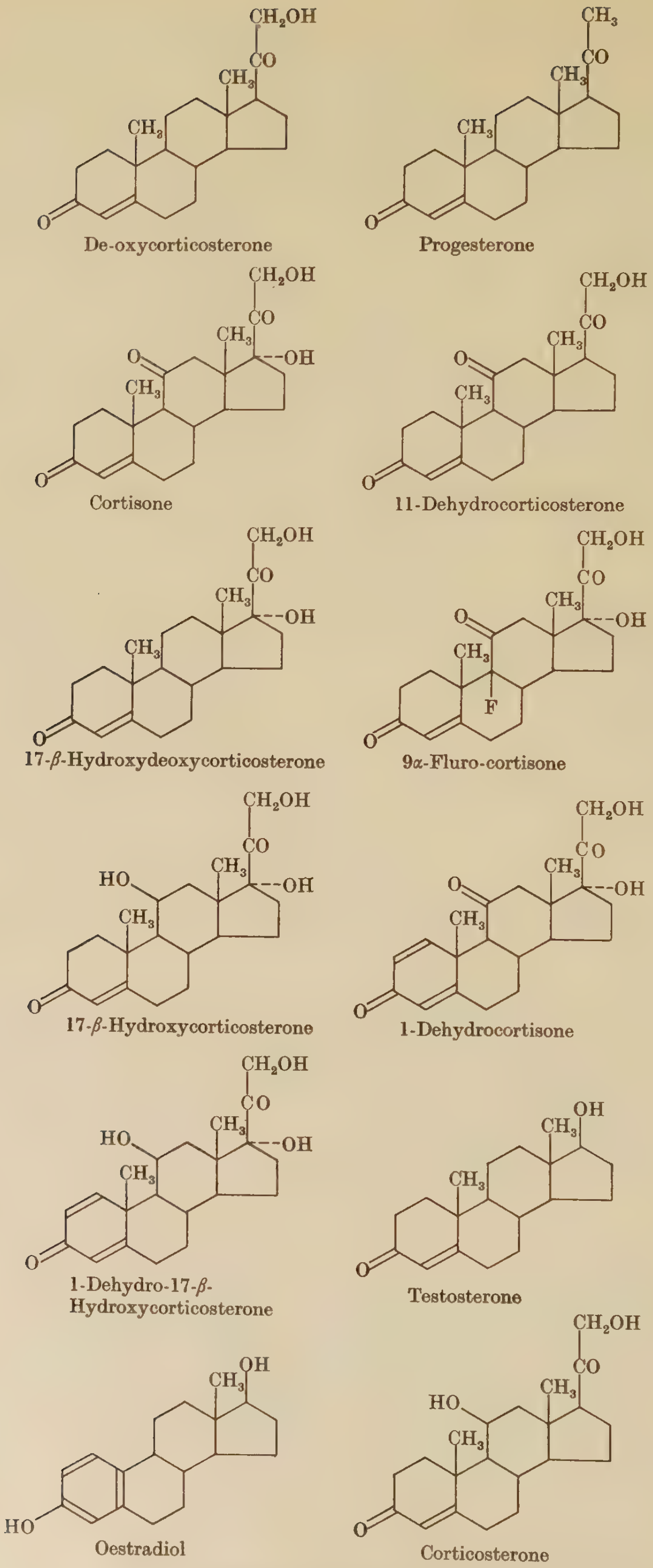
Indiana, U.S.A.) and cholesterol (Nutritional Biochemicals, Cleveland, Ohio, U.S.A.) were added as emulsions prepared as described previously (Conner, van Wagtendonk & Miller, 1953). These were tested at final concentrations ranging from 1×10^{-9} to 1×10^{-4}M.

All tubes were inoculated with 0·1 ml. of a 1/10 dilution of a 72 hr. culture of *Tetrahymena piriformis W.* The growth period was 96 hr. and the amount of growth was estimated as an increase in optical density measured in a Lumitron colorimeter with a 650 mμ filter.

All concentrations of each steroid were tested in triplicate and each experiment was repeated at least three times.

RESULTS

The steroids tested were readily divided into active and inactive groups. Inactive steroids were: testosterone, diethyl-stilboesterol, oestradiol, 1-dehydrocortisone and 1-dehydro-17-hydroxycorticosterone. The steroids which inhibited the growth of *Tetrahymena piriformis W* could be placed in a series ranging from slightly inhibitory to highly inhibitory. Table 2 gives the percentage and range of inhibition of growth for given concentrations of steroids.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De-oxycorticosterone

Progesterone

Cortisone

11-Dehydrocorticosterone

17-β-Hydroxydeoxycorticosterone

9α-Fluro-cortisone

17-β-Hydroxycorticosterone

1-Dehydrocortisone

1-Dehydro-17-β-
Hydroxycorticosterone

Testosterone

Oestradiol

Corticosterone

Fig. 1. Skeletal structures of the steroids.

The growth inhibition induced by progesterone and by corticosterone, respectively, and its annulment by stigmasterol is shown in Figs. 2 and 3.

Cholesterol seemed to be nearly as effective as stigmasterol in annulling growth inhibition in most experiments. Oestradiol had no effect.

Table 2. *Relative activity of various steroids in the inhibition of growth of* Tetrahymena piriformis W

Compound	Concentration (M)	Inhibition (range)* (%)
Progesterone	1×10^{-3}	20 (18– 24)
9 α-fluorocortisone	1×10^{-3}	18 (10– 25)
Cortisone†	1×10^{-3}	64 (50– 72)
11-ketoprogesterone	1×10^{-3}	67 (60– 80)
17-hydroxydesoxycorticosterone	1×10^{-3}	67 (54– 78)
17-hydroxy corticosterone	1×10^{-3}	75 (60– 90)
Desoxycorticosterone	$7{\cdot}5 \times 10^{-4}$	97 (90–100)
Corticosterone	$2{\cdot}5 \times 10^{-4}$	100
11-dehydrocorticosterone	$2{\cdot}5 \times 10^{-4}$	100

* Percentage inhibition is the average of nine determinations.

† Cortisone values given here are somewhat higher than reported previously (Conner & Nakatani, 1958). This difference is attributed to the sample of steroid used.

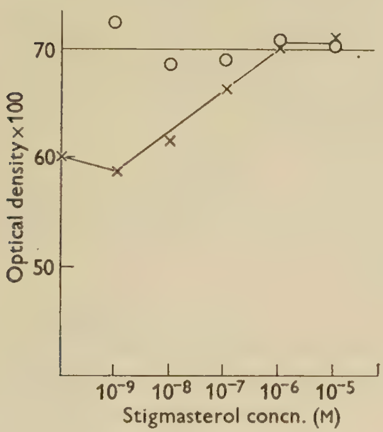

Fig. 2

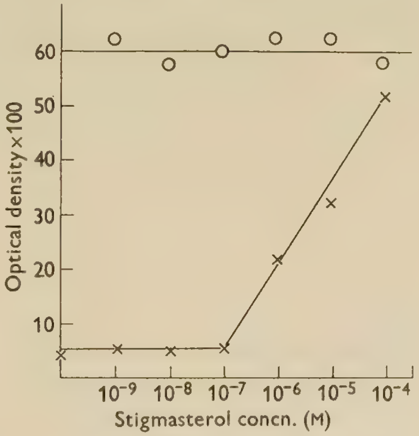

Fig. 3

Fig. 2. Growth response of *Tetrahymena piriformis* in presence of progesterone (1×10^{-3} M) with increasing concentrations of stigmasterol. With stigmasterol alone (○); with progesterone + stigmasterol (×).

Fig. 3. Growth response of *Tetrahymena piriformis* in presence of corticosterone ($2{\cdot}0 \times 10^{-4}$ M) and stigmasterol. With stigmasterol alone (○); with corticosterone + stigmasterol (×).

DISCUSSION

Comparison of the active and inactive steroids indicates the following specificities of effect:

(1) A side chain (C-20, -21) was necessary for growth inhibition. Progesterone (see Fig. 1 for structure of compounds) was inhibitory, while testosterone and estradiol were not.

(2) A hydroxyl grouping at C-21 enhanced inhibition. Progesterone was less inhibitory than deoxycorticosterone.

(3) Substitution of a keto or hydroxyl grouping at C-11 enhanced inhibition. Corticosterone was more inhibitory than deoxycorticosterone, 11-dehydrocorticosterone more than deoxycorticosterone, 11-keto progesterone more than progesterone.

(4) Substitution of a 17-β-hydroxy grouping lowered the inhibitory response. 11-Dehydrocorticosterone was more inhibitory than cortisone, and deoxycorticosterone more than 17-β-hydroxydeoxycorticosterone. 17-β-Hydroxycorticosterone was not available in amounts sufficient for testing to compare with corticosterone.

(5) Further unsaturation of the A ring lowered potency. 1-Dehydrocortisone and 1-dehydro-17-β-hydroxycorticosterone were less inhibitory than cortisone.

(6) Substitution in the B ring at position 9 lowered potency. 9-α-Flourocortisone is less inhibitory than cortisone. *Tetrahymena piriformis W* has been reported to contain steroid-like materials (McKee, Dutchen, Groupé & Moore, 1947; Seaman, 1950); from McKee's data it would appear this material is not cholesterol or stigmasterol. Perhaps these two sterols are converted to some physiologically active form. This supposition is strengthened by the findings of Holtz (1957) that a species of *Tetrahymena* required a sterol for growth, a requirement satisfied by cholesterol, β-sitosterol or stigmasterol. These organisms then differ from *Paramecium aurelia* which is unable to utilize cholesterol (Conner & van Wagtendonk, 1955). This may represent a greater metabolic lesion in the synthetic capacity of *P. aurelia*. The steroid isolated from *Tetrahymena piriformis* supported the growth of *P. aurelia* (Conner, 1957). Thus it would seem that a common metabolite or physiological form of the steroid exists in this group of protozoans.

The growth inhibition induced by certain of the steroids appears to be annulled by stigmasterol in a competitive fashion. Perhaps the configuration of the steroid inhibitor is related to the configuration of the steroid which is produced by the organism. It is striking that a carbonyl or hydroxyl grouping at carbon-11 greatly enhanced the degree of inhibition, while a carbon 17-β-hydroxyl substitution decreased it.

An action of steroids on metabolism has been reported for many types of organisms, organs, tissues, cells and cellular preparations. However, the probable *in vivo* mode of action for only one steroid, estradiol, has been elucidated (Talalay, Hudock & Williams-Ashman, 1958). The protozoans may provide further information about this. Investigations are at present being carried out to determine the mode of action of the inhibitory steroids on metabolism both *in vivo* and *in vitro*.

I wish to thank Dr L. Sarret for the generous supply of steroids used in this work, Dr O. Behrens for the stigmasterol, and Miss Jeanne Wenrich for her very competent technical assistance. This work was carried out with support from the National Science Foundation (Grant 2189) and The Public Health Service, National Institutes of Health (Grant 1065).

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The Morphology of *Nitrobacter winogradskyi*

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SUMMARY: The recently isolated organism of the second phase of nitrification is identified as *Nitrobacter winogradskyi* Buch. The organism reproduces by polar budding and possesses a distinct and easily demonstrated nucleus. The life cycle includes alternation of motile and non-motile stages. Motile cells possess a single flagellum which is 20 times longer than the cell body. The flagellum is laterally attached to the cell. The organism is related to the family Pasteuriaceae.

Until now only *Nitrobacter* spp. have been described as bringing about the second stage of nitrification. The morphological description of these organisms is incomplete. The mode of reproduction has not been described at all. Table 1 summarizes the data of those authors who have succeeded in pure culture isolation. The physiology of the isolated strains was the same and there was no doubt about the identity of these strains with that isolated by Winogradsky. Only Nelson (1931) differentiated his motile strain as *N. agilis* from the non-motile *N. winogradskyi*.

Table 1. *Nitrobacter morphology as described in literature*

Reference	Size (μ)	Form of the cells	Motility	Note
Winogradsky (1953)	0·5 × 1·0	Prolongated pear-shaped, the narrow end is curved like a 'beak'	Non-motile	Inside the cells the grains are stained
Wimmer (1904)	0·4 × 1·0	Rod-like	Motile	—
Gibbs (1919)	0·7 × 1·1	Oval	Non-motile	—
Prouty (1929)	0·8 × 1·0	Oval or spherical	Non-motile	—
Nelson (1931)	0·6 × 0·9	Rod with polar flagella	Motile	*N. agilis*
Kingma Boltjes (1936)	—	Ellipsoid	Non-motile	—
Engel & Skallau (1938)	0·4 × 1·0	Rod-like	Non-motile	Forms zoogloea
Meiklejohn (1953)	0·9 × 1·1	Oval	Non-motile	—
Zavarzin (1958)	0·4 × 1·1	Pear-shaped, budding. Single flagella	Motile in certain stages of development	Possess chromatin grain

An organism which brought about the second stage of nitrification was isolated by Zavarzin (1958); the results of study of this organism were reported at the 7th International Congress for Microbiology (Stockholm) by Imšenecki (1958). During the discussion the question arose whether it was possible to classify this organism as *Nitrobacter winogradskyi*. The present paper concerns the results of electronmicroscopic study of isolated strains.

METHODS

Two morphologically similar strains of Nitrobacter were isolated from rocks and soils of Middle Asia by means of a drop sprout method. Erlenmeyer flasks (50 ml.) containing Meiklejohn's (1953) medium were inoculated with small drops of enrichment culture. A drop was placed on the inner wall of a flask by a capillary pipette of such diameter that, on the average, a single organism would be found in each drop. About 3% of the subcultures were found to be pure.

The purity of the cultures was shown as follows. (1) By inoculation of nutrient media suitable for different physiological groups; about 20 different media were used. (2) By means of careful microscopic examination of cultures by phase-contrast microscopy. For the detection of *Hyphomicrobium vulgare* a method was developed which consisted in the inoculation of plates of Mevius medium solidified with agar. After incubation for 1 week at 28° white colonies were seen. Pure cultures of *Nitrobacter* sp. were cultivated in a liquid medium containing (g./l.) 2, $NaNO_2$; 0·15, $MgSO_47H_2O$; 0·15, KH_2PO_4; 0·03, $FeSO_47H_2O$; 0·5, $CaCO_3$; dissolved in tap water. No growth was observed on Bejerincks nitrite agar. The organisms from liquid cultures of different ages were centrifuged down and after fixation with osmium tetroxide and shadowing with chromium were examined by an electron-microscope.

RESULTS

The OsO_4 fixed Nitrobacter organisms appeared not to be so pleomorphic as live organisms examined by phase contrast. The most common forms were oval or coin-shaped; these were dominant in the lag phase. Sometimes something resembling nucleus could be recognized (Pl. 1, fig. 1), but it was much more convenient to study chromatin bodies by the light microscope.

At the narrow end of the cell there was a small swelling which made the cell pear-shaped (Pl. 1, figs. 2–4). A terminal swelling gradually increased in size and a distinctly recognizable bud was formed. The bud was separated from the mother cell by a small isthmus (Pl. 1, fig. 4). An elastic cell wall seemed common for both cells (Pl. 1, fig. 3). During the further development the bud reached the size of the mother cell (Pl. 1, fig. 5, left). At this stage it was possible to find some cells with a single flagellum attached laterally, apparently to the daughter cell. Sometimes the flagellum reached 20μ in length. Plate 1, fig. 5, represents an organism shortly before division, the second cell being the separated bud.

Motile Nitrobacter organisms were observed only during the logarithmic phase of growth, while the nitrite was not yet completely utilized. These organisms showed a rather slow swinging movement.

Thus, during multiplication, the bud was formed on the narrow end of the parent cell; this reached the size of the parent cell and then division took place. The bud possessed a single long flagellum.

The distribution of nuclear material in Nitrobacter cells was studied by means of the Feulgen reaction, double staining according to Peshkoff, staining with acetocarmine, Meyers haematoxylin, methyl green staining and by enzyme treatment. The nucleus in Nitrobacter cells may be demonstrated by staining with dilute fuchsin after acid hydrolysis which removed the chocolate-brown pigment. The results of applying all these methods were in agreement.

The mature cell and the bud from the very beginning of its formation contained the chromatic granules which corresponded in size to the sizes of the parent cell and the bud (Pl. 2, fig. 6).

In order to establish the identity of the isolated organism with that isolated by Winogradsky it was necessary to use the same methods of observation. Plate 2, fig. 7, represents the organisms from our culture stained with Ziehl fuchsin and Pl. 2, fig. 8, is an enlarged part of Winogradsky's photograph. The budding cells may be easily recognized on Winogradsky's micrograph made in 1891.

Consequently, it appears that the isolated strain of Nitrobacter may be classified as the species described by Winogradsky.

Systematic position of Nitrobacter

The reproduction by polar budding compels one to relate Nitrobacter to the family Pasteuriaceae order *Caulobacteriales*. The comparison of typical representatives of this group (Hyphomicrobium with Nitrobacter) is shown in Table 2.

Table 2. *Comparison of Nitrobacter and Hyphomicrobium*

Organism	Hyphomicrobium	Nitrobacter
Size (μ)	$0{\cdot}5 \times 2{\cdot}0$	$0{\cdot}4 \times 1{\cdot}1$
Shape	Egg- or rod-shaped	Pear-shaped
Appendages	Branching filaments ('stalks')	None
Mode of reproduction	Polar budding	Polar budding
Motility	The separated bud is motile	The separated bud is motile
Chromatinic bodies	Chromatin grains in mature cells and buds	Chromatin grains in mature cells and buds
Physiology	Oxidation of simple organic compounds	Oxidation of nitrite to nitrate
	Chemosynthesis (?)	Chemosynthesis

An essential difference between Hyphomicrobium and Nitrobacter is the absence from Nitrobacter of the long polar filament ('caulus' of Henrici & Johnson, 1935) at the tip of which the motile bud is formed by Hyphomicrobium. However, Henrici & Johnson (1935) pointed out that some representatives of Pasteuriaceae possess a very short stalk or none.

DISCUSSION

Isolates of organisms which carry out the second stage of nitrification, except for the observation of motility, which is difficult to detect without modern methods, correspond to Winogradsky's description. But there is here a contradiction with other observations. If we exclude the possibility of incomplete observations, two alternatives arise: (1) Different workers have studied different stages of life-cycles of Nitrobacter. Nelson and Wimmer described a logarithmic phase of growth when the organism is motile while other authors have a fixed stationary phase. (2) Organisms which carry out the second stage of nitrification which are similar physiologically and differ in morphology. This difference includes not only different degree of motility but a different form of the cells from coccoid to rod-like.

Until the mode of reproduction of different Nitrobacter strains, particularly *Nitrobacter agilis*, is studied, it is impossible to conclude which hypothesis is correct and how many species of Nitrobacter have been studied. If the second supposition is justified, we shall find the most interesting case of physiological convergence.

The authors wish to thank Professor A. Imšenecki for encouragement during the work. They wish to thank Professors M. Alexander and T. Y. Kingma Boltjes and Dr Jane Meiklejohn for valuable discussions.

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EXPLANATION OF PLATES

Plate 1

Figs. 1–5 electronmicrographs shadowed with chromium. The same enlargement for all five pictures

Fig. 1. Coin-like cell of Nitrobacter; beginning of bud formation.
Fig. 2. The second stage of bud formation, appearance of the swelling.
Fig. 3. Appearance of the isthmus between bud and mother cell.
Fig. 4. Pear-shaped cell with completely formed bud.
Fig. 5. To the left, bud shortly before the separation from the mother cell; to the right, separated bud. The flagellum attached laterally may reach 20μ in length.

PLATE 2

Fig. 6. The distribution of nuclear substance in 5-day cultures. Nuclei are stained like granules at the wide part of the cells. Stained with dilute fuchsin after pigment had been removed. Light microscope.

Fig. 7. Nitrobacter stained with Ziehl fuchsin. The organisms are oval, sometimes pear-shaped.

Fig. 8. Enlarged portion of centre of Winogradsky's micrograph (1953, Table 5, fig. 1). The budding is distinctly seen in some of the Nitrobacter organisms. The organisms indicated by arrows and numbers correspond to the electromicrographs of present paper.

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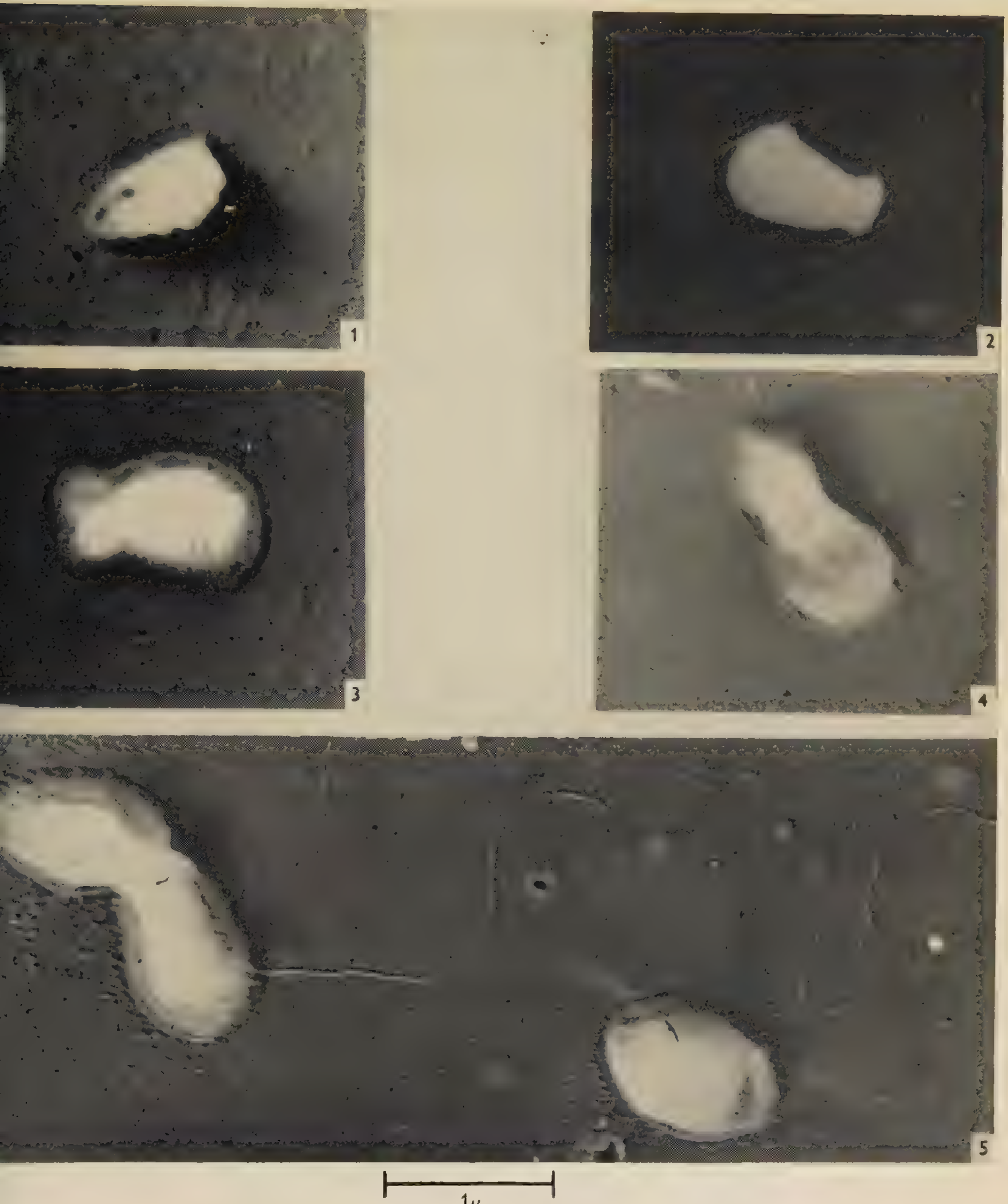
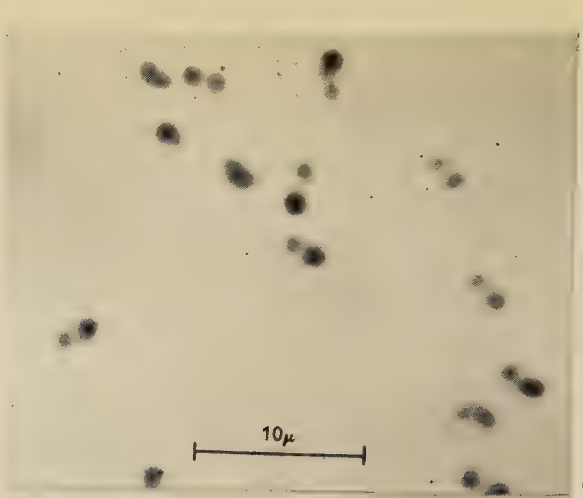


G. ZAVARZIN & R. LEGUNKOVA—NITROBACTER WINOGRADSKYI. PLATE 1

(*Facing p.* 190)



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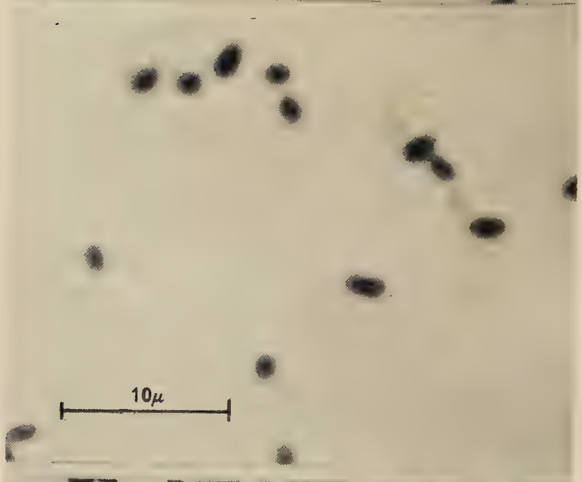

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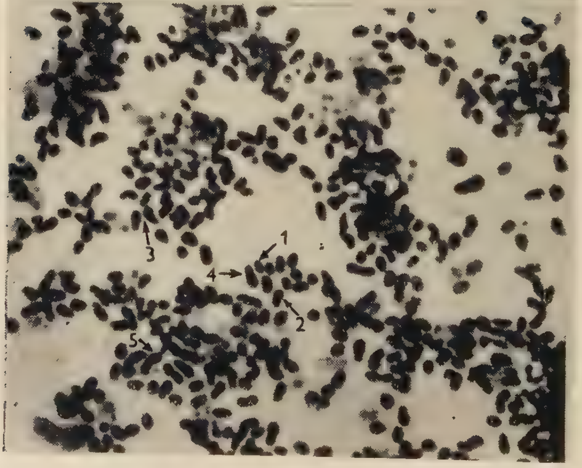

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G. Zavarzin & R. Legunkova—Nitrobacter winogradskyi. Plate 2

JENKIN, C. R. & ROWLEY, D. (1959). *J. gen. Microbiol.* 21, 191–202

Toxic Proteins from *Vibrio cholerae* and Water Vibrios which are Lethal for Mice

BY C. R. JENKIN AND D. ROWLEY

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SUMMARY: A toxic protein has been isolated from Inaba and Ogawa strains of *Vibrio cholerae* and from water vibrios by dissolving the bacteria in 2·5 M-urea and subsequent fractional precipitation with ammonium sulphate. This toxic protein accounted for the major portion of the toxicity of whole organism. Immunological and chemical data suggest that the toxic protein is the protein component of the Boivin antigen. The lipopolysaccharide endotoxins of the strains examined were relatively non-toxic as compared with the lipopolysaccharides from other Gram-negative bacteria, though in other biological and chemical properties they resembled the general class.

There is a marked tendency on the part of many research workers in the cholera field to assume that the manifestations of the disease are caused by a specific toxin which is liberated from the bacteria during growth. The proof of this hypothesis is difficult for two reasons. First, there is little precise information about the toxins of *Vibrio cholerae*, and much confusion exists as to the chemical nature and similarity of those substances which have been described. The second difficulty is that it has not been possible to produce a disease resembling cholera in experimental animals; therefore it is difficult to test toxins for their ability to reproduce the signs of the natural disease.

Boivin & Mesrobeanu (1935) isolated a toxic antigenic complex from *Vibrio cholerae* by extraction with cold trichloroacetic acid and characterized it as a 'glucolipid'. Similar Boivin antigens have been extracted from many Gram-negative bacteria and have been shown to possess the 'O'-antigenic specificity of the parent organisms. It is not clear in the case of *V. cholerae* how completely this glucolipid complex could account for the toxicity of whole organisms. Bernard & Gallut (1945) confirmed the existence of a toxic Boivin-type antigen and reported a second small-molecular toxin which was later purified to some extent by Gallut & Grabar (1945). Burrows (1944) claimed to have extracted a highly toxic phospholipid from *V. cholerae* by using hot ethanol. This material contained, on occasion, 500 mouse LD 50 doses/mg. and, moreover, caused an increase in permeability to water of the small intestine of rabbits, when tested *in vitro*. This effect on permeability was abolished by using intestines from animals immunized with *V. cholerae*. Freter (1956) described the isolation of two toxic fractions from *V. cholerae*, one acid-soluble and the other insoluble in HCl at pH 3·5. The acid-insoluble material had a mouse LD 50 of approximately 500–750 μg. and appeared to be a lipoprotein; the acid-soluble toxic material had a mouse toxicity of

50–100 μg. and on analysis resembled the Boivin type-antigen (4–6 % N; 1·5–2 % P).

At least five potentially different toxins have therefore been reported in the literature. Their relationship to each other is obscure and their quantitative importance in accounting for the overall toxicity of *V. cholerae* is unknown, since none of these authors gave any exact data on the percentage recovery of toxicity in their fractions.

In the present work an attempt has been made to repeat these earlier investigations in a quantitative manner. A new method by which a potent toxic protein can be extracted in good yield from vibrios is described. The immunological relationships between these various toxins will be described later.

METHODS

Organisms. Strains Ogawa 5596, Inaba 4693 and Hikojima 7270 were obtained from the National Collection of Type Cultures (Colindale, London), whilst strains Ogawa 161, 162, 163 and water vibrios 53, 54 and 55 were kindly supplied by Dr K. Bhaskaran of the Central Drug Institute, Lucknow, India.

Maintenance of cultures. All cultures were freeze-dried in 10 % (v/v) serum +5 % (w/v) glucose in nutrient broth. One freeze-dried culture was used to inoculate 6 nutrient agar slopes for routine use. After growth the agar slopes were kept at 4° and no slope was opened on more than ten occasions. The Inaba and Ogawa strains were tested periodically against homologous antisera.

Growth and harvesting of the organisms. Organisms were grown for 48 hr. at 37° on nutrient agar (pH 8·6) and harvested in saline in an ultraviolet irradiated cabinet. The harvested suspension was passed through glass wool to eliminate pieces of agar.

Toxicity tests. For these tests mice weighing 18–20 g. were injected intraperitoneally. They were observed for 72 hr. and then discarded. LD 50 doses were calculated by the method of Reed & Muench (1938). A minimum of 5 mice were used at each dose level and for the more important materials such as the toxic protein, the total numbers of mice tested at each level amounted to more than fifty (Table 8).

Centrifugation. Centrifugation at 1200–5000 *g* was obtained in an M.S.E. 'Magnum' refrigerated centrifuge; at 6000–20,000 *g* in a Serval SS 2 ultracentrifuge; at 20,000–78,000 *g* in a Spinco ultracentrifuge.

Ultrasonic disintegration. This was carried out in a 2 kW. Mullard ultrasonic disintegrator type E. 7590 A with a frequency output of 25 kc./sec. In all cases the apparatus was used with maximum power output at maximum efficiency, which was found by trial and error to occur when the stainless steel cup contained 30–40 ml. suspension of bacteria at a concentration of 30 mg. dry wt./ml. This operation was conducted at 0°.

Chemical analyses. Nitrogen estimations were by the macro-Kjeldahl method, with the apparatus of Markham (1942) for the distillation.

Phosphorus determinations were made by a modification of the Fiske & SubbaRow (1925) method. *Total carbohydrate* was determined as described by Seifter, Dayton, Novic & Muntyler (1950). *Protein* was determined by the Folin–Ciocalteau reagent as described by Kabat & Mayer (1948) with bovine albumin as a standard. *Lipid A* was extracted as described by Westphal & Lüderitz (1954). *Methylpentoses, hexoses* and *pentoses* were determined by the methods of Dische & Shettles (1948) and Dische, Shettles & Osnos (1949). *Nucleic acid* was estimated by measuring the absorption of a solution containing 250 μg. fraction/ml. by the Beckman u.v.-spectrophotometer at 260 mμ., comparing with a yeast nucleic acid standard.

Electrophoresis. The conjugated toxic protein toxin was examined for purity by: (*a*) starch-gel electrophoresis by the method described by Smithies (1955); (*b*) zone electrophoresis on Whatman no. 1 paper at 6 V./cm. in borate buffer (pH 7·3; ionic strength 0·05) and in veronal buffer (pH 8·6; ionic strength 0·075); (*c*) moving-boundary electrophoresis carried out on a 1 % (w/v) solution of the toxic protein in a Perkin–Elmer modification of the Tiselius apparatus at pH 7·4 in a phosphate buffer containing $NaH_2PO_4.H_2O$ (0·460 g./l.), $Na_2HPO_4.12H_2O$ (5·95 g./l.) and NaCl (8·78 g./l.).

Biological tests on the lipopolysaccharides. These materials were tested for the ability to produce the following effects: (*a*) non-specific immunity by the method of Rowley (1956); (*b*) Schwartzman effect as described by Schwartzman (1937); (*c*) pyrogenic response. The rectal temperatures of rabbits were taken during 2 hr. to ensure a constant zero reading. Three rabbits were each given 0·05 μg. lipopolysaccharide intravenously in 1 ml. pyrogen-free saline. Temperatures were taken at intervals during 5–6 hr. after injection.

RESULTS

Alcohol extraction (after Burrows)

No toxic materials could be extracted by hot alcohol from the three *V. cholerae* serotypes, whilst the residue from these alcohol extractions was always non-toxic.

Hydrochloric acid extraction

It was sometimes found by following Freter's (1956) method of extraction that 40–50 % of the toxicity of whole organisms could be extracted. However, this did not always occur; occasionally very little toxicity was extracted and on testing the residue it was found that considerable loss of toxicity had occurred during incubation with the hydrochloric acid. Even in these cases where toxicity had been extracted it was always lost during the subsequent purification steps, which involved solvent extraction and precipitation from hot ethanol.

Extraction with trichloroacetic acid

Preliminary experiments showed that the toxicity of *Vibrio cholerae* whole organisms decreased markedly when heated at 100° for 60 min. or by exposure to strong trichloroacetic acid solutions. For this reason the extraction was performed on vibrio suspensions (killed at 75° for 30 min.) by using 0·05 M-trichloroacetic acid, the whole extraction being conducted during ultrasonic disintegration for 60 min. at 0°. The mixture was centrifuged at 3500 *g* for

60 min. to recover undisrupted bacteria and other material, the supernatant fluid from this was centrifuged for 60 min. at 16,000 *g* and the resulting supernatant fluid from this spun at 78,000 *g* for 2 hr. The latter centrifugation sedimented the Boivin-type macromolecular complex. All these fractions were dialysed and the dry weight measured, before testing for toxicity in mice. The results of a typical experiment with the *V. cholerae* strain Ogawa 5596 (Table 1) show that *c.* 16 % of the original toxicity was recovered in the Boivin antigen with a toxicity of 5 mouse LD 50/mg. Similar extraction experiments with the Inaba 4693 and Hikojima 7270 strains did not yield any material which would sediment at 78,000 *g* even when 0·5 M- or 0·1 M-trichloroacetic acid was used for extraction. Analysis of the toxic complex obtained from the Ogawa strain 5596 showed it to contain 35 % protein. The Boivin antigen extracted from *V. cholerae*, Ogawa strain 5596, was relatively heat labile; heating at 100° for 60 min. led to considerable loss in toxicity (Table 2). The heat lability of this fraction is surprising since endotoxic complexes from other Gram-negative bacteria are remarkably heat stable.

Table 1. *Extraction of* Vibrio cholerae, *Ogawa strain* 5596, *with* 0·05 M-*trichloroacetic acid in the ultrasonic apparatus for* 60 *min.*

		Recovery	
	Weight (g.)	% by weight	% of initial toxicity
Starting material	3·45	—	—
Residue from extraction	2·13	62	35
Deposit 16,000 *g*	0·36	10·4	39
Deposit 78,000 *g*	0·05	1·4	16
Total recovery	—	73·8	90

Table 2. *Stability of the Boivin-type toxin from* Vibrio cholerae, *Ogawa strain* 5596, *at* 100°

Injected dose of Boivin toxin (μg.)	Time (min.) of heating at 100°		
	0	30	60
	No. mice dead/no. mice injected		
500	7/8	6/9	3/9
250	5/9	4/9	0/9

Extraction with phenol/water

Suspensions of the three serotypes of *Vibrio cholerae* were killed by heating at 100° for 10 min. and were extracted at a concentration of 20 mg. dry wt. organisms/ml. water layer by the phenol/water method of Westphal, Lüderitz & Bister (1952). The data from typical extractions on these serotypes are collected in Table 3. The weights of the various fractions are those of the residual non-dialysable material, since the phenol had been removed by prolonged dialysis. The final yields of purified lipopolysaccharides varied between 0·5 and 2·0 % of the original dry weight, which is similar to the amounts obtained from other Gram-negative organisms. However, both the toxicity and the recovery of toxicity were extremely low in all cases by comparison with a typical extraction of *Escherichia coli* (Table 3).

Chemical analysis of the lipopolysaccharides. Deposits at 78,000 *g*, which by their previous extraction history were presumptive lipopolysaccharides, from various vibrio strains were analysed for nitrogen, phosphorus, sugar and Lipid A content (Table 4).

Biological properties. Although the toxicities of lipopolysaccharides from the vibrio strains were low by comparison with those from most other Gram-negative organisms, further tests showed that these compounds possessed biological properties typical of the general class.

Table 3. *Extraction of the three serotypes of* Vibrio cholerae *and* Escherichia coli 2380 *with* 45 % (*w*/*v*) *phenol in water*

	Inaba 4693	Ogawa 5596	Hikojima 7270	*E. coli* 2380
Wt. of starting material (g.)	15·7	3·52	10·5	4·6
No. LD 50 doses	6240	1408	1750	1840
Wt. in water layer (g.)	5·14	0·40	3·1	1·2
Recovery of toxicity (%)	16·4	—	—	—
Wt. in phenol layer (g.)	3·12	0·57	1·8	0·8
Recovery of toxicity (%)	<5	<2·7	<7	—
Wt. in residue (g.)	4·7	1·9	2·3	1·4
Recovery of toxicity (%)	7·5	9	7	15
Wt. of lipopolysaccharide (g.)	0·39	0·02	0·18	0·11
Recovery of toxicity (%)	11	0·7	5	74

Stimulation of non-specific immunity

Groups of mice were given intravenously, in 0·2 ml. of saline, 50µg. or 10µg. lipopolysaccharide from *Vibrio cholerae* Ogawa, Inaba and Hikojima strains. The control mice were given 0·2 ml. of saline only, by the same route. After 48 hr. the mice were challenged intraperitoneally with a virulent strain of *Escherichia coli* 145 (10^5 organisms in 0·2 ml. of 1·75 % (w/v) solution of hog gastric mucin). The results and a comparison made with a group of mice given 10µg. lipopolysaccharide from *E. coli* 2380 are given in Table 5. In all cases lipopolysaccharide derived from the three serotypes of *V. cholerae* stimulated non-specific immunity to an extent approaching that caused by *E. coli* 2380.

Schwartzman effect. This phenomenon was described by Schwartzman in 1937. When endotoxin is injected into the skin of a rabbit and 24 hr. later a further amount is given intravenously, a haemorrhagic necrosis rapidly develops at the site of the first injection. In a typical experiment the abdomen of a rabbit was shaved and four squares drawn on the prepared area of skin. Into two of these 5µg. Ogawa lipopolysaccharide in 0·2 ml. saline were injected subcutaneously, whilst the other two sites were injected with 0·2 ml. saline only. After 24 hr. the rabbit was given 20µg. of the same lipopolysaccharide in 0·2 ml. intravenously, and the prepared skin squares examined after 18 hr. Haemorrhagic necrosis developed only at those sites previously injected with lipopolysaccharide.

Table 4. *Collected chemical and biological data on the lipopolysaccharides from different vibrios*

Organism	Yield of lipopoly-saccharide (%)	Mouse LD 50/kg. (mg.)	Recovery of toxicity as lipopoly-saccharide (%)	Nitrogen (%)	Phosphorus (%)	Lipid A (%)	Presence of		
							Methyl-pentose	Hexose	Pentose
Vibrio cholerae									
Ogawa 5596	0·67	50	2·5	2·1	2·0	54	+	+	+
Ogawa 161	2·07	25	16	nd	nd	50	nd	nd	nd
Ogawa 162	2·4	15	21	2·2	2·3	60	nd	nd	nd
Inaba 4693	1·83	37	14	2·4	1·9	22	+	+	+
Hikojima 7270	1·85	100	4·4	2·4	2·4	34	+	+	+
Water vibrio 53	2·35	25	16	2·4	2·0	65	+	+	+
Water vibrio 54	3·33	25	16	2·5	2·2	25	+	+	+
Water vibrio 55	2·39	25	16	2·25	2·1	36	+	+	+
Escherichia coli 2380	2·25	2·5	50	2·1	1·8	12	+	+	−

nd = analysis not done.

Pyrogenicity. The samples of Inaba, Ogawa & Hikojima lipopolysaccharides all proved to be highly pyrogenic in rabbits; 0·05 μg. of any of them produced a 2° F. rise in temperature which was comparable to the temperature rise obtained with a similar amount of lipopolysaccharide from *Escherichia coli* 2380.

Table 5. *Stimulation of non-specific immunity by lipopolysaccharides*

The mice were challenged after 48 hr. with *Escherichia coli* 145 (10^5 organisms + mucin).

Lipopolysaccharide	Intravenous dose (μg.)	Treated mice. No. deaths/no. injected
V. cholerae, Inaba 4693	50	1/8
	10	5/8
V. cholerae, Ogawa 5596	50	3/8
	10	4/8
V. cholerae, Hikojima 7270	50	2/8
	10	3/8
Escherichia coli, 2380	10	1/8

The control groups received 0·2 ml. saline and were challenged with *E. coli* with the treated groups, after 48 hr. All the mice died in each set of controls.

Table 6. *Antigenic specificity of lipopolysaccharides from* Vibrio cholerae, *Ogawa and Inaba strains*

Maximum dilution to produce agglutination or precipitation

	Ogawa 'O' antiserum	Inaba 'O' antiserum
Ogawa organisms (2 hr. at 100°)	1/4000	1/500
Ogawa lipopolysaccharide	1/256	1/16
Inaba organisms (2 hr. at 100°)	1/250	1/4000
Inaba lipopolysaccharide	1/8	1/256

Antigenic specificity. Lipopolysaccharide from Ogawa and Inaba strains of *Vibrio cholerae* were titrated against homologous and heterologous antisera which had been produced by injecting rabbits with a suspension of whole organisms heated for 2 hr. at 100°. Both samples of lipopolysaccharide were used in the precipitin tests at a concentration of 500 μg./ml. The results (Table 6) showed that the lipopolysaccharides were specific for the parent organisms from which they had been isolated.

Urea and ammonium sulphate fractionation

Urea possesses the property of making proteins soluble and has been successfully used in the isolation of polysaccharides from *Mycobacterium tuberculosis* (Stacy, Kent & Nassau, 1951) and endotoxin complexes from *Salmonella typhi* (Walker, 1940). In all the following extractions living bacterial cultures were used. Preliminary tests showed that the bacteria were rapidly dissolved by the urea solution during disintegration in the ultrasonic apparatus at a constant low temperature. Dialysis of the opalescent solution deposited approximately 50 % of the starting material, which weight for weight had a similar toxicity to that of the suspension of whole organisms. This deposit could be recycled.

By ammonium sulphate fractionation of the dialysed supernatant fluid, toxic fractions of high protein content were precipitated. The sharpness of separation was greatly improved by fractionation in the presence of 2·5 M-urea. It was important throughout the extraction and fractionation procedures to control carefully the temperature of the solutions; all extractions, precipitations and centrifugations were carried out at 0°. The toxic fractions were found to be more stable in the presence of phosphate ion; resuspension in saline or distilled water led eventually to loss in toxicity.

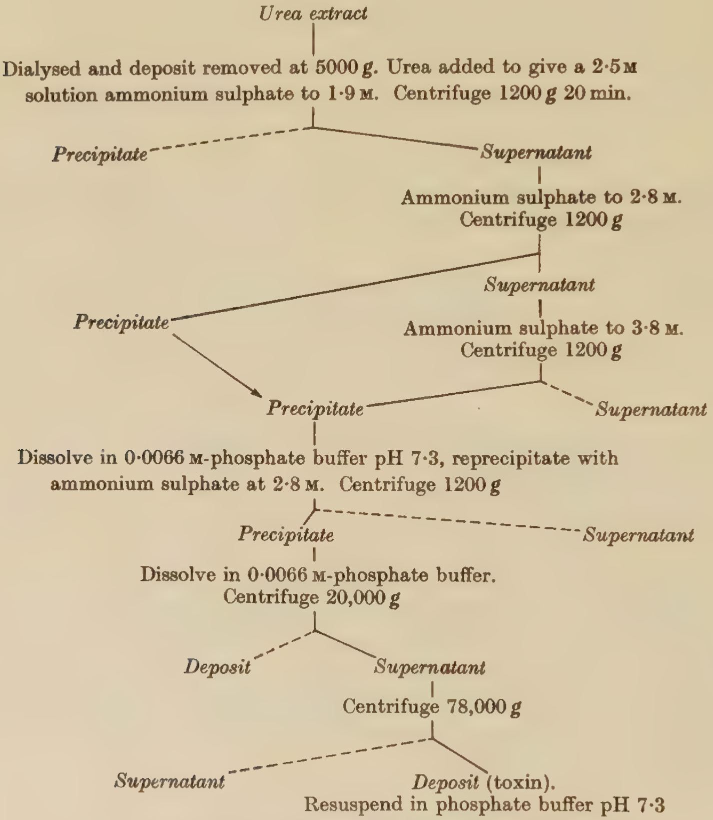
Urea extract
Dialysed and deposit removed at 5000 g. Urea added to give a 2·5 M solution ammonium sulphate to 1·9 M. Centrifuge 1200 g 20 min.
Precipitate
Supernatant
Ammonium sulphate to 2·8 M. Centrifuge 1200 g
Supernatant
Precipitate
Ammonium sulphate to 3·8 M. Centrifuge 1200 g
Precipitate
Supernatant
Dissolve in 0·0066 M-phosphate buffer pH 7·3, reprecipitate with ammonium sulphate at 2·8 M. Centrifuge 1200 g
Precipitate
Supernatant
Dissolve in 0·0066 M-phosphate buffer. Centrifuge 20,000 g
Deposit
Supernatant
Centrifuge 78,000 g
Supernatant
Deposit (toxin). Resuspend in phosphate buffer pH 7·3

Fig. 1. Extraction of a protein toxin from *Vibrio cholerae*

The final extraction method was as follows. To a suspension of living vibrios in saline at a concentration of 30 mg. dry wt./ml., solid urea was added 2·5 M. The suspension was agitated in the ultrasonic apparatus at 0° for 5 min., during which time the turbidity greatly decreased. The opalescent suspension was dialysed against running tap water at 10° for 18 hr. and the contents of the dialysing bag centrifuged at 5000 g. Solid urea was again added to the clear amber-coloured supernatant fluid to 2·5 M and this solution was precipitated at 0° successively with neutral saturated ammonium sulphate at 1·9 M, 2·8 M and 3·8 M saturation (Fig. 1). The precipitates at 2·8 M and 3·8 M saturation were highly toxic and were combined for further purification. The combined precipitates were dissolved in 0·0066 M phosphate (pH 7·3) to give a colourless opalescent solution which was reprecipitated with neutral saturated ammonium sulphate to 2·8 M. The resulting precipitate was again dissolved in 0·0066 M-phosphate (pH 7·3) and centrifuged at 0° in the Spinco first at 20,000 *g* for 1 hr. and finally at 78,000 *g* for 2 hr. The deposit obtained at 78,000 *g* represented the purified toxic protein which contained 10–20 mouse LD 50 doses/mg. and accounted for between 10 and 45% of the original toxicity, depending on the strain extracted. The results of a typical extraction of *Vibrio cholerae*, Ogawa strain 5596, are given in Table 7.

Table 7. *Combined results of four separate extractions of toxic protein from* Vibrio cholerae, *Ogawa strain* 5596

	Starting material	Deposit at 78,000 *g*
Weight (g.)	43·7	0·570
Weight recovery (%)	—	1·3
LD 50 (average)	2·2 mg.	75 μg.
Limits	1–3 mg.	50–100 μg.
Recovery of toxicity (%)	—	25
Protein (%)	—	100
Phosphorus (average %)	—	1·5
Limits (%)	—	1·3–1·7
Nitrogen (average %)	—	13·8
Limits (%)	—	13·6–14·0

Different strains of the same serotype, different serotypes or even water vibrios all yielded similar toxic protein fractions by this method. The results of some of these extractions are collected in Table 8.

The toxic protein obtained from Ogawa strain 5596 was examined for purity by starch-gel, zone and moving-boundary electrophoresis. By the latter two methods the material moved as one component which suggested a high degree of purity. However, no movement was detected in starch gel, which suggests that the toxic protein is of high molecular weight and is not contaminated with protein of smaller molecular size.

Chemical properties of the toxic protein

The toxicity of the protein was completely destroyed by heating at 100° for 10 min. The toxic proteins from the Ogawa and Inaba strains were less labile at 60°, about 50% of the toxicity still remaining after heating for 60 min. This is somewhat surprising in view of the lability of the toxic material to changes in pH and temperature during its extractions, though like many other proteins its stability to physical changes may increase with degree of purification. Incubation of the toxic protein with proteolytic enzymes such as trypsin, papain and pepsin, in an effort to break it down into smaller toxic units, led only to complete loss of toxicity. About 50% of the toxicity was lost as a result of freeze-drying the toxic protein. The toxicity was best retained by suspending the protein toxin in 0·0066M-phosphate (pH 7·3) and keeping the solution at 2°.

Table 8. *Accumulated data on the protein toxin extracted from various vibrios with 2·5M-urea*

	Ogawa 162	Ogawa 163	Water vibrio 55	Water vibrio 54	Inaba 4693
Weight of starting material (g.)	4·0	3·0	2·8	3·4	7·2
No. LD 50 doses	2000	1200	920	1100	3000
% weight recovery in deposit at 78,000 *g* (%)	1·0	0·66	2·85	1·17	0·33
LD 50 dose (μg.)	100	80	200	100	80
Recovery of toxicity in protein (%)	22·5	20·8	43·3	36·3	10·3
Total recovery of toxicity in all fractions (%)	70	83·3	86·6	82	60
Protein in toxin (%)	100	100	100	100	100
Carbohydrate in protein (%)	3	1·2	1·7	1·2	nd

nd = analysis not done.

Recovery of toxic protein from Boivin antigen

Previous experiments had shown that the toxicity of the Boivin antigen was heat-labile and acid-labile. It seemed possible therefore that the toxic protein isolated might in fact be the protein component responsible for the toxicity of this macromolecular complex.

Boivin antigen (90 mg.) obtained by trichloroacetic acid extraction (containing 38% protein; mouse LD 50, 200μg.) was taken up in 2·5M-urea and fractionated with ammonium sulphate as previously described. The precipitate obtained at 3·8M weighed 32 mg. and contained 10 mouse LD 50 doses/mg. Analysis of the precipitate showed it to contain 92% protein and 8% carbohydrate.

These results show that a toxic protein may be isolated from the toxic Boivin antigen. From immunological data the protein toxin isolated from the whole organisms and that isolated from the Boivin antigen appear to be identical (Jenkin, unpublished).

DISCUSSION

Three toxic fractions have been prepared from *Vibrio cholerae* by different chemical methods. The protein toxin isolated by urea and ammonium sulphate fractionation appears to account for the major portion of the toxicity of whole organisms. This protein toxin occurs in the strains of *V. cholerae* and in the water vibrios tested. It is desirable to examine any relationship that might exist between the present protein toxin and those toxins of *V. cholerae* isolated previously, particularly in the work of Gallut & Grabar (1945) and of Freter

(1956). Gallut & Graber described a dialysable polypeptide toxin which was present in greatest amount when autolysis of a heavy suspension of *V. cholerae* proceeded for 72 hr. When the autolysis was stopped after 4 hr. very little, if any, of this toxin was found. The toxicity of the supernatant fluids due to the high molecular 'gluco-lipidique' Boivin complex was greatest after 4 hr. autolysis, and subsequently decreased when lysis continued for longer than this period, with the appearance of the small molecular toxin. With the progressive loss in toxicity they noted that the nitrogen content of 'gluco-lipidique' complex also diminished, and it was suggested that the polypeptide toxin was split from this large polymolecular complex by action of proteolytic enzymes. The suggested derivation of the polypeptide toxin from the Boivin antigen, taken in conjunction with the finding that the protein toxin isolated from *V. cholerae* in this work is part of this antigen, makes it seem likely that the polypeptide toxin of Gallut & Grabar and the toxic protein *V. cholerae* are similar. The only apparent reason to call the material polypeptide, was its ability to pass through a membrane of such pore size as to allow the passage of haemoglobin.

The acid-insoluble toxin isolated by Freter (1956) is similar in many ways to the toxic protein we isolated. The low toxicity of Freter's protein may have been due to the method of isolation and purification, which involved solvent extraction and precipitation from hot ethanol. All these procedures would certainly lead to loss in toxicity of the protein toxin isolated in this paper. When repeating Freter's method of acid extraction any extracted toxicity was always lost during the subsequent purification steps. It may be suggested that the toxic proteins of Gallut & Grabar and of Freter are similar to the toxic protein we isolated by urea and ammonium sulphate treatment, but that the latter method is far gentler and retains most of the toxicity of this fraction.

It must be emphasized that in the present work only those fractions of *Vibrio cholerae* responsible for the lethal effect of whole organisms injected into mice have been considered. Since death from any of these toxic fractions is rarely, if ever, accompanied by diarrhoea, it is by no means probable that these toxins cause the gross change in intestinal permeability which seems to be the most important clinical feature of cholera.

We wish to thank Dr T. Brogan and Miss B. Haywood for technical advice on electrophoresis and Mr W. Ali for the chemical data on *Escherichia coli* 2380 lipopolysaccharide. One of us (C.R.J.) is grateful to the Wright–Fleming Institute for the award of a Fleming (Pfizer) Fellowship during the period of this work, which forms part of a Ph.D. thesis submitted to the University of London.

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Attempts to Obtain Gram-Negative Rods from Staphylococci Treated with Penicillin

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SUMMARY: Gram-negative bacilli could not be obtained from the Oxford strain of *Staphylococcus aureus* nor from 15 other strains of *S. aureus* in the presence of benzylpenicillin.

In recent communications Crawford & Abraham (1957) and Briggs, Crawford, Abraham & Gladstone (1957) reported interesting morphological and physiological phenomena associated with the Oxford strain (NCTC 6571) and a penicillinase-producing strain (D3R) of *Staphylococcus aureus*. When the two strains were cultivated for less than 24 hr. in broth in the presence of penicillin, bacitracin or cephalosporin C and bacitracin, respectively, they were found to give rise to Gram-negative rods. Subcultures of the resulting Gram-negative rods differed from the original staphylococcal strains, not only in colonial morphology and biochemical reactions but in phage susceptibility and animal pathogenicity as well. Under certain cultural conditions it was reported possible to obtain re-emergence of the Gram-positive cocci from the Gram-negative rod cultures. The obvious implications of these apparently distinct reversible changes in morphological and physiological properties of staphylococci prompted us to undertake similar studies.

METHODS

In addition to the Oxford strain, 15 other strains of *Staphylococcus aureus*, mostly of recent human origin, were used in the present work. Each of the strains was plated out three consecutive times on brain heart infusion agar (Difco) to verify morphological and tinctorial homogeneity. The strains were all coagulase-positive and were completely inhibited by penicillin in concentrations of 0·038–0·076 μg./ml., as determined in a twofold tube dilution assay with inocula of 50–100,000 organisms.

One-tenth ml. of a *c.* 18 hr. broth culture of each strain was inoculated into 60 ml. brain heart infusion broth (Difco) contained in a screw-capped Erlenmeyer flask. After incubation for 2–3 hr. at 37°, when the cultures were in the logarithmic phase and the broth was slightly cloudy, sodium benzylpenicillin (1670 units/mg.; batch 29293, Glaxo Laboratories Ltd.) was added to give a final concentration of 1 μg./ml. With continued incubation the organisms lysed and the broth remained clear for periods ranging from 2 to 21 days, when the cultures again became cloudy. The flasks were opened, subcultures made to blood agar plates and smears made during the first 24 hr. of incubation and later when the cultures became cloudy. Aeration of duplicate cultures during incubation was effected by means of a rotary action flask shaker.

The strains of *Staphylococcus aureus* which were used are listed below. The number of times each strain was cultured and examined for its ability to produce Gram-negative rods in the presence of penicillin is denoted by two numbers following the strain; whether the cultures were aerated or stagnant is indicated by an A or an S: Oxford strain 15 A, 6 S; strain 209 P (ATCC 6538 P) 9 A, 6 S; strains (BB 1), (DC 4), (JC 1), (LB 2), (NL 2), (399), (825), (852), (932), (944), (3406), (3559), (3565) and (3582) each 6 A, 6 S.

RESULTS

Sixteen penicillin-sensitive strains of *Staphylococcus aureus* were cultured in the presence of benzylpenicillin for a total of 204 times (108 aerated, 96 stagnant); only Gram-positive cocci were found in the stained smears of the cultures and only staphylococcal colonies grew out on subculture to blood agar plates. The penicillin and broth controls included with each series of tests remained clear and showed no organisms on stained smear or on subculture.

Our inability to induce the emergence of Gram-negative rods in cultures of the Oxford and 15 other strains of staphylococci with penicillin is in sharp contrast to the observations of the Oxford workers. The reasons for this discrepancy in results, particularly with the Oxford strain, remain unknown. Though the Oxford workers interpreted the appearance of Gram-negative rods in their cultures as morphological and physiological variants of staphylococci, the possibility of an inadvertent introduction or the presence of a previously unrecognized contaminant is of foremost concern here and deserves comment. Inapparent and prolonged coexistence of two bacterial species is not an uncommon phenomenon; Barringer & Dack (1935) documented such an arrangement with the bacteroides and several other micro-organisms. The Oxford workers attempted to eliminate the possibility that their staphylococcal strains were contaminated by picking single organisms by micro-manipulation for initiation of cultures. However, seeing individual small Gram-negative rods in the living unstained state might be difficult and it is conceivable that contaminants might not be detected on such a visual examination.

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Further Observations on the Relationships between Gram-Negative Rods and Staphylococci Grown in the Presence of Penicillin

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SUMMARY: It has not been found possible to repeat the work of Briggs *et al.* (1957) in which Gram-negative rods were isolated from cultures of staphylococci treated with penicillin, and staphylococci were subsequently recovered from cultures of these rods when the latter were grown in the absensce of penicillin. No flaw in technique has been revealed which would enable the earlier results to be readily explained in terms of contamination.

In a series of experiments designed to compare the effects of penicillin, cephalosporin C and bacitracin on *Staphylococcus aureus* (NCTC 6571) growing in aerated broth, Crawford & Abraham (1957) observed that Gram-negative rods frequently appeared and multiplied after most of the staphylococci had undergone lysis. When this phenomenon was first encountered we suspected that the Gram-negative organism was a contaminant which had entered an environment favourable for its selection. But when the phenomenon subsequently recurred we recalled that several groups of workers had previously reported that staphylococci underwent a transformation to Gram-negative rods on 'training' to resist high concentrations of penicillin, and that this transformation was reversible (Klimek, Cavallito & Bailey, 1948; Gale & Rodwell, 1948; McVeigh & Hobdy, 1952). We therefore decided that the phenomenon deserved further study under conditions more stringent than those employed by any of the groups which had described it previously. Single cocci were isolated from a culture of the Oxford staphylococcus. Gram-negative rods were isolated from a culture, derived from one of these single-coccus isolates, which had undergone partial lysis in the presence of penicillin; 12 separate rods were thus isolated. After serial subculture in the absence of penicillin 10 of these isolates gave rise to staphylococci of the parental phage type (Briggs, Crawford, Abraham & Gladstone, 1957). The strict precautions taken to exclude contaminants and the controls designed to show that the precautions were adequate were described in detail in that paper.

We concluded from these results that a transformation of the staphylococcus to a rod was responsible for the phenomenon. The evidence for this conclusion, in particular the repeated recovery of staphylococci from cultures of the Gram-negative organisms under conditions in which the staphylococci had no striking selective advantage, seemed at the time to be highly convincing.

During the summer of 1957 re-wiring of the department necessitated the suspension of work for three months and the cultures available were freeze-dried. Professor S. Elek and Dr G. R. F. Hilson then informed us that they had obtained Gram-negative rods under conditions similar to those that we had used, but were unable to demonstrate a reversion of any strains of our rods to staphylococci. In an attempt to provide them with a freshly isolated strain of our Gram-negative rod we repeated our original experiments in November 1957 under conditions which were apparently identical with those used earlier. On this occasion, however, in repeated experiments we obtained no Gram-negative rods from the Oxford staphylococcus, nor from 14 other coagulase-positive strains of *Staphylococcus aureus*. Several strains of *S. aureus* were also grown on gradient plates containing 0·1 unit penicillin/ml. (Szybalski, 1953) and colonies which formed near the inhibition zone were examined and subcultured. Although many of these colonies contained a large proportion of Gram-negative cocci, and the Gram-positive cocci present were frequently enlarged and distorted, no rods were observed.

Attempts were then made, by the methods used earlier, to recover staphylococci from the cultures of Gram-negative rods which had been stored in the freeze-dried state. These attempts also were unsuccessful. On several occasions small numbers of Gram-positive cocci were observed in cultures of the rod which had been grown in the absence of penicillin in Mitchell flasks (Mitchell, 1949), but these cocci could not be isolated.

We have been unable to uncover any flaw in technique which would allow us to account for our earlier results in terms of adventitious contaminants. Paine & Daniel (1959) suggest that the isolation of single rods was not in fact achieved and that staphylococci were carried over (presumably on ten successive occasions) into the cultures of rod-shaped organism from which they were subsequently recovered. One of us (S.B.), who personally carried out micromanipulations for the isolation of single rods, considers that this hypothesis is untenable.

Nevertheless, the present failure by Hilson & Elek (1959), by Paine & Daniel and by ourselves to repeat completely the experiments of Briggs *et al.* (1957) must cast doubt on the authenticity of the transformation which these experiments appeared to demonstrate. Since the phenomenon has now been observed by four groups of workers it may well be re-encountered. In that event we hope that our own experience will be helpful in further attempts to establish its true explanation.

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An Investigation into the Development of Gram-negative rods in Penicillin-treated Cultures of *Staphylococcus aureus*

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SUMMARY: Attempts were made to obtain Gram-negative rods from the Oxford strain of *Staphylococcus aureus* in the presence of benzylpenicillin. Two such isolations were made in 476 test cultures and one in 192 controls. The difference is not significant. No reversion to coagulase-positive staphylococci could be demonstrated of a rod-shaped Gram-negative organism previously reported as showing this effect. The question of the presence of Gram-negative rods in staphylococcal cultures exposed to penicillin is discussed and it is considered that the phenomenon is due to contamination.

The isolation of Gram-negative rods from a strain of *Staphylococcus aureus* serially subcultured many times in the presence of increasing concentrations of penicillin was first reported by Klimek, Cavallito & Bailey (1948). Similar observations were described by Gale & Rodwell (1949) and by McVeigh & Hobdy (1952). More recently, it was reported that Gram-negative rods could be found at various times during the development of individual cultures of *S. aureus* in the presence of benzylpenicillin, cephalosporin C or bacitracin (Crawford & Abraham, 1957), and this phenomenon was studied further, with special precautions against contamination, by Briggs, Crawford, Abraham & Gladstone (1957). They used only benzylpenicillin and were able to isolate Gram-negative rods with some regularity from overnight cultures, even when the cultures had been derived from single-cell isolations of the staphylococcal strain. Single-cell isolations were also made of the Gram-negative rods, and during subsequent maintenance of these cultures under different conditions Gram-positive cocci resembling the original strain were once more isolated from them.

There can be no doubt that it is possible to isolate Gram-negative rods from penicillin-treated cultures of *Staphylococcus aureus*: the finding is, however, open to two interpretations. One is that contamination becomes manifest through the selective action of the drug against the original inoculum; the second, that the rods are in some way directly derived from the staphylococci by mutational or adaptive mechanisms. In view of the special precautions taken by Briggs *et al.* and their report of the reappearance of staphylococci in bacillary cultures, their work provides the strongest evidence hitherto that the second interpretation may be the correct one. In view of the fundamental importance of the problem an attempt to confirm their findings was made.

METHODS

Bacterial strains. Freeze-dried cultures of the Oxford strain of *Staphylococcus aureus* (NCTC 6571) and of one substrain of a Gram-negative bacillus ('G-11' of Briggs *et al.*) were kindly sent by Miss Briggs.

Media. The basic medium used throughout was Bacto (Difco) heart infusion broth (HIB). This was used instead of the tryptic digest broth referred to originally by Briggs *et al.* since we learnt that in their later work more frequent isolations of Gram-negative rods occurred in HIB (Briggs, personal communication). For solid media, HIB was used with 2% (w/v) New Zealand agar added (HIA). Salt agar was made by adding 7% (w/v) sodium chloride to HIA; for a small number of experiments a stock 'saturated salt broth' was made by saturating HIB at room temperature with sodium chloride, allowing the crystals to settle, and using the supernatant. Various conventional media and methods were used in studying the properties of Gram-negative rods: horse blood agar, MacConkey's and Koser's media and peptone water fermentation bottles were prepared according to Mackie & McCartney (1953) and tests for indole and hydrogen sulphide production, and Methyl-red and Voges–Proskauer reactions were carried out as described in the same text. Ammonia production was tested for as described in *Topley and Wilson's Principles* (1955). Anaerobic cultures were incubated in McIntosh and Fildes jars. Total bacterial counts were made by examining a suitable dilution of a culture in an 'Improved Neubauer' counting chamber; for viable counts the method of Miles & Misra (1938) was used. For most of the work, HIB was distributed in 9 ml. amounts in 25 ml. screw-capped bottles ('universal containers'), in 'Pyrex' glass 'T'-tubes (Crawford & Abraham 1957), or in a modification of the latter referred to as 'F'-tubes (Fig. 1). The latter maintained cultural conditions identical with those in T-tubes but permitted inoculation by syringe and needle through a rubber vaccine cap and so obviated withdrawal of the cotton-wool plug at any time after sterilization. The metal caps of the bottles were also perforated to allow entry with a syringe needle through the rubber disks beneath.

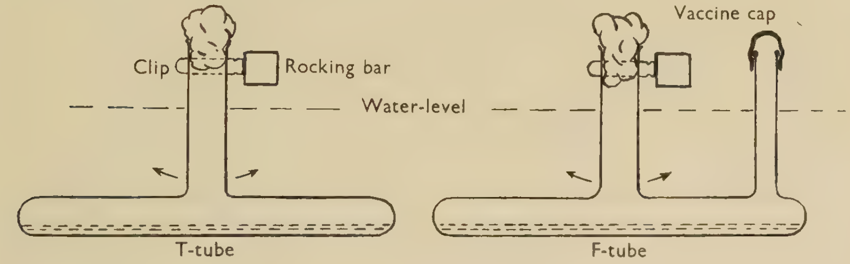

Fig. 1

Sterilization of bottles and tubes was by autoclaving at 120° for 15 min. The media were always sterilized in the containers in which they were to be used.

The central mouths of the T- and F-tubes were closed by means of a cotton-wool plug, and during autoclaving, sterility testing, and storage were covered by brown paper caps held on with rubber bands. All media were tested for sterility by incubation at 37° for 2 days and then at room temperature for at least 2 days. Syringes were tested for good fit of plungers, lubricated with liquid paraffin, and placed, with needles mounted, in test tubes or boiling tubes, being sealed at the necks by several layers of surgical gauze bandage wrapped around the barrel of the syringe. They were sterilized in the hot-air oven by exposure to 160° for $1\frac{1}{2}$ hr.

Inoculation of T-tubes and bottles was by platinum loop or sterile graduated pipettes, except as indicated below. Where a 'closed' technique was being followed, with inoculation by syringe and needle, the following procedure was adhered to: the rubber surface was rubbed liberally with spirit (95 % ethanol + 5 % water), which was then ignited and the needle passed through the dying flame and into the rubber. To minimize damage to the rubber, no needles thicker than no. 15 (0·7 mm. diam.) were used.

Shaking of cultures. The term 'aerated' is used to describe cultures in T- or F-tubes which were rocked at 42 cycles/min. through an arc of approximately 70°, partially submerged in a water bath at 37°; 'rotated' cultures were those clipped to a turntable set at 45° to the vertical, turning at 30 r.p.m. and incubated in a 37° incubator; and 'stagnant' cultures were those kept in a 37° incubator without any agitation.

Penicillin solutions. Benzylpenicillin (Glaxo Laboratories Ltd.) was used as the sodium salt. It was dissolved in distilled water and diluted to a concentration of 100 μg./ml. for the earlier attempts to isolate Gram-negative rods. This solution was sterilized by passage through a '5 on 3' sintered glass filter and distributed by conventional methods in 2 ml. amounts in sterile bijou (5 ml.) screw-cap bottles. The filter was tested by subsequently passing through it 10 ml. of an overnight culture of a highly penicillin-resistant strain of *Staphylococcus aureus* with an excess of fresh broth and incubation for 2 days at 37°; no growth resulted. In the later 'closed' experiments a solution of 20 μg./ml. was filtered through a Seitz EK filter (similarly tested subsequently) and distributed in 8 ml. amounts by removal through a rubber diaphragm with a syringe into sterile screw-cap bottles. The solutions were stored at −15°, a sample bottle being taken occasionally for titration of its penicillin content to detect any deterioration.

Isolation of Gram-negative rods. Penicillin-treated staphylococcal cultures showing abnormal turbidity (all were slightly turbid) were examined by Gram staining and subcultured with a platinum loop on to HIA plates containing 1 μg. penicillin/ml. The latter were incubated for 48 hr. if necessary. Individual colonies developing on penicillin-agar plates were picked off on to penicillin-free plates and purified by two repetitions of the conventional single-colony subculture. Gram staining and testing for penicillin-resistance by streak inoculation on to penicillin agar completed the preliminary examination, and several HIA slopes were inoculated, incubated and stored at 4° for further investigations. The other fluid cultures showing only normal turbidity were similarly subcultured on to penicillin agar before being discarded. They were not examined routinely by Gram staining since it was very soon found that the short diplobacillary form of the G-11 strain of Briggs *et al.* (1957) could not be reliably distinguished from the many Gram-negative and swollen diplococci present in penicillin-treated cultures of staphylococci.

*Erythromycin and bacitracin sensitivities of G-*11. These were compared with those of the Oxford staphylococcus by means of a disk diffusion method.

RESULTS

Isolation of Gram-negative rods from penicillin-treated staphylococcal cultures

The freeze-dried culture of the Oxford staphylococcus was suspended in 0·25 ml. HIB, and a loopful plated on HIA for purification. The remainder of the suspension was spread on a penicillin agar plate. All colonies developing after overnight incubation on HIA resembled *Staphylococcus aureus*, though poorly pigmented, and all those tested were composed of Gram-positive cocci and gave the slide coagulase reaction. Two successive single colony subcultures were made to complete the purification, and then a colony was subcultured on to several HIA slopes to provide stock cultures for the experiments described below. These cultures were not opened after inoculation until required for use, when they were checked by Gram staining and coagulase testing. The penicillin agar plate inoculated with the remainder of the freeze-dried culture was sterile after 4 days' incubation at 37°, showing that there had been no admixture with organisms resembling G-11.

The general pattern of inoculation and incubation was the same for all three groups of experiments. In preparation for each day's experiment, a stock culture on HIA was inoculated into a bottle of HIB and incubated overnight. From this stagnant culture sets of 12 or 24 containers of HIB (differing in each group) were inoculated and incubated with agitation at 37° to an arbitrary standard turbidity, when penicillin was added and the incubation continued overnight. After further stagnant incubation for 48–72 hr. they were tested for the presence of Gram-negative rods. The required degree of turbidity before penicillin addition was determined by naked-eye comparison with a 'standard' provided by Miss Briggs. This consisted of one of her cultures, from a similar experiment at the same stage, killed and preserved with a few drops of formalin. It contained about 2×10^7 staphylococci per ml. A few cell counts were done on growing cultures which had reached this degree of turbidity and these also gave results of the same order of magnitude.

Experimental group 1—'*rotated cultures*'. In this group, the stagnant overnight culture was inoculated into screw-cap bottles of HIB with a graduated pipette in amounts varying from 0·1 to 0·5 ml. in different batches of 12. The periods of incubation with rotation to standard turbidity varied from 1·75 to 2·25 hr. Penicillin (100μg./ml.) was added in 0·1 ml. amounts, also with a pipette, to give a final concentration of 1μg./ml.

After overnight incubation with rotation, lysis by penicillin had produced a moderate reduction in turbidity of the cultures which changed little during the subsequent stagnant incubation. Gram staining at this stage showed a mixture of Gram-positive and Gram-negative diplococci with some swollen forms; as already mentioned, these abnormal staphylococci could not be reliably differentiated by their appearance from the short diplobacilli often seen in actively-growing cultures of G-11. Cultures on HIA invariably showed large numbers of surviving staphylococci. In all, none of 144 broth cultures carried out in this manner showed abnormal turbidity, nor did they yield Gram-negative rods on penicillin agar.

This cultural technique differed a little from that of Briggs *et al.* (1957): its suitability for allowing the emergence of organisms resembling G-11 was subsequently tested. Identical batches of 12 cultures were set up, inoculated with *Staphylococcus aureus* and treated with penicillin as before, but with the addition, either at the same time as the penicillin or 1 hr. later, of a very small inoculum, standardized by total and viable counts, from a young culture of G-11: usually about 0·1 ml. of a 10^{-7} dilution. In five different batches the estimated average viable inoculum varied from 0·3 to 1·0 bacillary unit; of the total of 60 cultures, 36 grew G-11 on subsequent incubation, showing that this method of culture would permit the emergence of small numbers of this strain.

Experimental group 2—'*aerated cultures*'. In view of the failure of the first group of experiments to yield Gram-negative rods an attempt was made to follow the methods of Briggs *et al.* (1957) in every minute detail affecting cultural conditions. Batches of 12 T-tubes were inoculated with 0·2 or 0·3 ml. of overnight cultures, rocked in the water bath to the required turbidity, and treated with penicillin as before.

(*a*) Four batches of cultures, a total of 48 T-tubes, were tested. Abnormal turbidity was seen in one culture after the overnight aerated incubation, and a Gram-negative rod was isolated on the penicillin-agar plate from this culture. This organism was designated 'G-A' and investigations on it are reported below. Up to this point, since test cultures had yielded no Gram-negative rods, negative controls had not been used; it had been realized, however, that they would become necessary in the face of a positive finding. Four batches of T-tubes of HIB were therefore processed in such a way as to mimic every procedure used in the test cultures, except that the staphylococcal inoculum was lacking: a bottle of HIB was 'inoculated' with a sterile platinum loop (instead of from a staphylococcal stock culture), and incubated overnight; from it, 0·2 ml. amounts were added to each T-tube, which, after the usual interval of aerated incubation, received its due amount of penicillin and further incubation. Such control tubes were, of course, normally crystal clear throughout and subcultures were sterile. However, one tube showed a slight turbidity at the end of the full period of incubation and Gram staining and culture on penicillin agar revealed a second Gram-negative rod 'G-B'.

(*b*) Further sterile precautions appeared to be desirable. At this time, 'settle plates' of HIA exposed to the air in the room in which the T-tubes were being inoculated showed an occasional colony resembling G-B, although this and other Gram-negative bacillary cultures were never handled in the room.

It was thought likely that contamination might occur through air-borne organisms settling on, or near, the cotton-wool plugs at any time after removal of the protective brown-paper cap, and being sucked into the tube when the plug was removed for addition of the penicillin. A modification of procedure was therefore devised whereby the cotton-wool plug was covered with a sterile metal cap ('oxoid') immediately the brown-paper cover was removed. To introduce a pipette into the tube, the operator lifted off the cap by hand, and an assistant pulled out the plug with sterile forceps, this procedure being reversed after pipetting had been completed. After the penicillin had been added, only the plug was replaced, after flaming. Since the cap fitted loosely, and was in place only during the short period before penicillin addition, there was no significant effect on the amount of aeration of the cultures. Size of inoculum, period of preliminary incubation, and amount of penicillin added to each tube were as in (*a*). Batches of 12 were set up at a time, and these were alternated with batches of negative controls which had received an 'inoculum' of sterile broth instead of staphylococci. In all, 132 test cultures, and 144 controls, were completed. All of the latter remained sterile; one tube in the ninth batch of the test series yielded a culture of Gram-negative rods on penicillin agar, though it had shown no excess turbidity at 48 hr. This organism was labelled G-C.

Experimental group 3—'*aerated cultures*' *with* '*closed*' *technique.* In order to diminish further the risks of contamination the preparation of materials for use in this series and the methods of handling the cultures themselves differed considerably from what has been described in regard to the first two series.

Stock cultures of the Oxford staphylococcus: a screw-capped bottle of HIB was inoculated by means of a platinum loop from a stock culture on an HIA slope, with the least possible exposure to the open air. After overnight incubation at 37°, stock broth cultures were made from it by taking out 0·8 ml. with a 1 ml. syringe and injecting 0·1 ml. amounts into broth bottles, which were then incubated overnight and stored at 4°. The remainder of the original broth culture, about 8 ml. in volume, was centrifuged at 4000 *g* for half an hour and the entire deposit spread on an agar plate containing 1 μg. penicillin/ml.: no growth occurred after 48 hr. at 37° and 3 further days at room temperature. The overnight broth culture required for each batch of tests was made from a stock broth culture with syringe and needle as described. The inoculation of F-tubes was carried out with a 5 ml. syringe and needle, each tube being entered through the rubber cap and receiving 0·2 ml. of overnight culture. After the tubes had been inoculated from the latter, it was checked for the presence of Gram-positive cocci and coagulase activity. Benzylpenicillin solution (20 μg./ml.), was prepared and distributed as described in 'Methods'. For each batch of cultures, 6 ml. of penicillin solution was taken from a bottle with a 10 ml. syringe, and 0·5 ml. injected into each F-tube, the final concentration being once more 1 μg./ml.

These precautions were intended to ensure that the materials used in the tests, once sterilized, or, in the case of the staphylococcal cultures, tested for the absence of Gram-negative rods, should not suffer exposure to the open air greater than that occasioned by the presence of a capillary meniscus of fluid inside a syringe needle. The general pattern of testing was as before, batches of 12 F-tubes being inoculated, incubated in the water bath with aeration for about 2 hr., and injected with penicillin before further aerated and stagnant incubation. No control tubes were set up in this series. None of 152 cultures yielded Gram-negative rods.

*Characteristics of strains G-*11, *G-A*, *G-B* and *G-C*

*Strain G-*11. The freeze-dried culture of this provided by Miss Briggs was re-constituted with a few drops of HIB and plated out on HIA. After overnight incubation at 37° it was possible to detect the fact that two types of colony were present. Both types were about 2 mm. diameter, smooth and creamy in appearance, and butyrous in consistency, but one was very slightly more translucent than the other. After standing at room temperature for a further 24 hr., distinction between the colonial types was easier, as the more translucent ones showed a moderate peripheral mucoid change ('Schleimwallbildung'); the other type of colony had merely become slightly more opaque. The translucent colonies predominated and were made up of Gram-negative diplobacilli; the other colonies were those of a staphylococcus giving a positive slide coagulase reaction and with a penicillin-sensitivity similar to that of the Oxford staphylococcus. Three serial conventional single-colony subcultures on HIA of the Gram-negative organism produced growths having all the appearances of a pure culture, in which colonies containing Gram-positive cocci could no longer be found. A single colony was used to inoculate several HIA slopes for stock cultures of G-11. Most of the properties investigated are listed in Table 1. The main points of note are that it is a freely growing non-motile Gram-negative rod which is an obligate aerobe, grows on MacConkey's medium, is non-saccharolytic and utilizes citrate in Koser's medium. Its properties in relation to penicillin are not shown in the table but are of some interest.

Penicillin sensitivity. Serial doubling dilutions of penicillin in HIB were made (4 ml. volumes in universal containers) and each inoculated with one drop of an overnight HIB culture of G-11. After 24 hr. incubation at 37° growth equal to the control took place in 4μg./ml. with partial growth in 8μg./ml. At 48 hr. full growth had extended to 16μg./ml. To investigate the effect of inoculum size, serial tenfold dilutions of a young (incubated for 4 hr. and faintly turbid) HIB culture of G-11 were made, and HIA plates containing 16, 8, 4, 2 and 0μg. penicillin/ml. were inoculated with 0·02 ml. drops of each dilution. Each plate also received a loopful of undiluted culture and a spot inoculum from a slope culture of the Oxford staphylococcus as a control of penicillin activity. After 3 days' incubation, growth from the various dilutions was equal to the control on concentrations of penicillin up to 4μg./ml., but there was none on plates containing higher concentrations. The loopful of undiluted culture, however, produced growth on the 16μg./ml. plate.

Penicillinase production. This 'inoculum effect' suggested penicillinase production, and a penicillin-destroying activity of G-11 was in fact demonstrated. A heavy inoculum of the strain was streaked in the centre of a HIA plate containing 1 μg. penicillin/ml. and incubated overnight, producing a streak of luxuriant growth. A loop carrying a portion of a solid culture of the Oxford staphylococcus was then streaked at right angles up to and through the G-11 growth with a steady movement. After further overnight incubation, growth of the staphylococcal inoculum was found to a zone extending 3 mm. out from the G-11 streak, demonstrating considerable reduction in effective penicillin concentration near to the latter. To confirm this finding, 10 ml. HIB containing 8 μg. penicillin/ml. was inoculated with 0·1 ml. of a fully grown culture of G-11 in HIB and incubated overnight. The densely turbid culture was centrifuged to deposit most of the bacilli, and the supernatant inoculated

Table 1. *Properties of the Oxford staphylococcus and of the Gram-negative organisms G-*11, *G-A*, *G-B and G-C*

	Oxford staph.	G-11	G-A	G-B	G-C
Motility ...	—	—	—	—	—
Broth 37°, 48 hr.	Even turbidity		Pellicle	Even turbidity	
Growth on:					
HIA, 48 hr., colony diam. and appearance	3 mm. creamy	3 mm. creamy translucent	3 mm. greenish metallic	0·5–1mm. greenish mucoid	
Blood agar, 37°, 48 hr.	+++	+++	+++	+++ (6 days)	
18°, 48 hr.	++	++	+	+++ (6 days)	
6°, 48 hr.	+	+	−	− (6 days)	
Blood agar, anaerobic, 37°	++	−	−	−	
NaCl agar, 48 hr.	++	−	−	−	
MacConkey, 48 hr.	+	+++	+++	+ (7 days)	
Citrate (4 days)	−	+	−	−	
Coagulase	+	−	−	−	
M.R.	+	−	−	−	
V.-P.	+	−	−	−	
Indole	−	−	−	nt	
Ammonia from peptone	+	−	−	nt	
H_2S	−	−	−	nt	
Acid from:					
Glucose	+	−	−	−	
Lactose	+	−	−	−	
Maltose	+	−	−	−	
Sucrose	+	−	−	−	
Mannitol	+	−	−	−	
Dulcitol	−	−	−	−	
Erythromycin	Sens.	Res.	nt	nt	
Bacitracin	Sens.	Res.	nt	nt	

+ signs indicate approximate amount of growth or a positive result.
− signs indicate no growth or negative result.
nt = not tested.

with the Oxford staphylococcus. Free growth of the latter on further incubation, as shown by Gram staining and subculture on salt agar, demonstrated penicillin destruction by G-11.

Strain G-A. This organism was isolated from an aerated staphylococcal culture of experimental group 2a and was a non-motile Gram-negative rod with a colonial appearance on HIA very similar to that of G-11. Its characteristics are also described in Table 1; it differed from G-11 in producing slightly greenish metallic colonies on HIA, a stringy growth and pellicle in HIB, instead of a diffuse turbidity, and in not utilizing citrate.

Strains G-B and G-C. G-B was isolated from an aerated broth control tube of experimental group 2a, and G-C from a penicillin-treated staphylococcal culture of group 2b. These organisms appeared very similar to each other, and to have several characters (Table 1) contrasting markedly with those of G-11 and G-A. Both were slowly growing non-motile Gram-negative rods, producing greenish mucoid colonies reaching 2 mm. diameter on blood agar only after 6 days and growing just as well at room temperature (18°) as at 37°.

Attempts to recover staphylococci from cultures of G-11

It has already been mentioned that the reconstituted freeze-dried culture of G-11 gave rise to a mixture of Gram-negative rods and a penicillin-sensitive coagulase-positive staphylococcus. However, in the course of routine purification, the first subculture of a colony of the Gram-negative organism showed only two colonies of Gram-positive cocci and each of two further subcultures showed none. From the last of these, stock HIA slopes of G-11 were set up, and in the course of investigations involving the use of this organism, many further subcultures on HIA and in HIB were made. Gram-positive cocci were not found in any of these.

The selective action of sodium chloride was also used in order to try to discover the presence of staphylococci in cultures of G-11. Preliminary experiments showed that G-11 was inhibited, with some sterilization, by sodium chloride in approximately 8 % (w/v) concentration in HIB, while small inocula of the Oxford staphylococcus grew readily under these conditions. Batches of 12 screw-capped bottles of HIB (10 ml.) were inoculated with 0·2 ml. of an overnight broth culture of G-11 and rotated for $2\frac{1}{2}$ hr. at 37°. To each of the moderately turbid cultures 3 ml. of sterile saturated salt broth was then added with a graduated pipette, and incubation with rotation continued overnight. A loopful was taken from each culture on to salt agar plates which were incubated for 48 hr. Four batches of twelve were completed in this way. In one culture of the first batch dense turbidity had developed after overnight incubation, and this was found to contain a mixture of albus and citreus types of staphylococci, both coagulase-negative, and the latter with optimal growth at room temperature. All of the other 47 cultures were negative. Settle plates of the room in which this work was being carried out showed that similar staphylococci were the predominant aerial flora.

DISCUSSION

The results reported above may be summarized as follows: out of 144 agitated, and 332 aerated (total 476), cultures of the Oxford staphylococcus treated with benzylpenicillin in the logarithmic phase of growth, only two yielded Gram-negative rods (G-A and G-C). These differed in a number of properties from each other and from the organism G-11 of Briggs *et al.* (1957). Of 192 control tubes lacking a staphylococcal inoculum, one yielded a Gram-negative rod (G-B). In approximately a hundred subcultures (after routine purification) of G-11, in a high proportion of which the selective action of sodium chloride was employed, no staphylococci resembling the Oxford strain were found. These findings, therefore, represent only a partial confirmation of the work of Briggs *et al.* (1957) in regard to the apparent conversion of staphylococci to Gram-negative rods, and none in regard to the reversion of the latter to coagulase-positive staphylococci.

It appears to us that the problem of the transformation of a staphylococcus into a Gram-negative rod requires consideration under two headings. The first is whether standard bacteriological techniques, even with the added precautions that have been used, are sufficiently reliable to exclude contamination with certainty. The second involves an assessment of the evidence for a genetic relationship between the two strains.

Bacteriological procedures for the maintenance of pure cultures involve a chain of techniques: a chain whose strength is only that of its weakest link. Heat treatment of media and glassware provides the strongest link, with the least probability of contamination, and that provided by filtration with subsequent testing of the filter must be nearly as strong. It is very difficult to judge the risks of contamination incurred in the transference of the bacterial strain itself, which necessarily is exposed to a variable extent during the process. There is no indication in the reports of earlier workers (Klimek *et al.* 1948; Bellamy & Klimek, 1948; Gale & Rodwell, 1949; Kawamata & Shigeto, 1955) that they used any precautions against contamination beyond those customarily employed. Moreover, since they used methods of serial transfer, it is likely that the risk of contamination in their work was high. They used no controls which would allow any estimate of its probability. With the aid of an inoculation cabinet and other precautions Briggs *et al.* (personal communication) were able to avoid contamination in approximately 300 controls; in our work one contaminant appeared in 192 control cultures.

We are not convinced that single cell selection by micro-manipulation (de Fonbrune, 1949) lessens the risk of contamination very much. It provides a reasonable guarantee that a culture with properties generally resembling those of the parent has arisen from a single member of the latter, but our own experience with the method suggests that since the growth developing from the single cell is subsequently handled by standard methods, contamination is possible at that and later stages. The 'syringe and rubber diaphragm' method of inoculation used by us in Experimental group 3 seemed on theoretical grounds likely to diminish the risk of contamination, and no rods were found in 152 cultures; a very much larger number of tests would however be necessary to establish statistically the advantage of this method.

The success in general of standard methods for the maintenance of pure cultures depends not so much on the absence of contamination, as on the fact that a contaminant can rarely become manifest in the absence of an agent acting selectively in its favour. Conversely, it is common knowledge from work in the field of bacterial genetics that when such selection is applied, a single resistant mutant (or contaminant) can be detected with ease. It is our view that the methods so far employed in this field (including our own) have not been shown to be sufficiently refined to allow contamination to be excluded as an explanation for the emergence of Gram-negative rods from penicillin-treated cultures of *Staphylococcus aureus*. The evidential value of this emergence therefore depends on whether it occurs significantly more frequently in test cultures than in the controls. In our series there was no statistically significant difference. In the earlier work of Briggs *et al.* (1957), bacilli were found only in the test cultures with none in the controls, but they have been unable to confirm this (Briggs, Crawford, Abraham & Gladstone, 1959), and Paine & Daniel (1959) have also been unable to isolate Gram-negative rods.

It is a theoretical possibility that the Gram-negative rods are derived from the staphylococci, but the probability of this has to be viewed in the light of existing knowledge on bacterial genetics. There are no hard and fast rules about the number of characteristics by which a mutant can differ from its parent and still be accepted as such rather than as a new species. However, the resistant mutant isolated after the action of a selective agent is likely to differ in only a few characters, and with each new unrelated character there is a greatly reduced probability of it being a mutant. If the characters are not linked, the probability is the product of the individual mutation rates. When several characters are altered simultaneously (even if their individual rates are high) the mathematical probability of such an occurrence requires a parent population of astronomical size. The greater, therefore, the divergence from the parent, the greater the likelihood that a different species is being investigated: that is, contamination has to be postulated. Briggs *et al.* (1957) tabulate a series of comparisons between the Oxford staphylococcus and their organism including characteristics of morphology, sugar fermentation, protease and coagulase activity, haemolysin production, phage typability and carriage, and penicillin and polymyxin sensitivities. They differ in almost every particular. Our investigations confirm and supplement these data, findings of note being that G-11 utilizes citrate and shows much greater resistance to erythromycin and bacitracin than the staphylococcus. We also showed G-11 to possess penicillin-destroying activity, a result contrary to the findings of Briggs *et al.* and possibly due to differences in the techniques used to demonstrate the effect. Our additions to the list of properties investigated show a further divergence in character between the Oxford staphylococcus and G-11; in fact, it is notable that the staphylococcus and the Gram-negative rod share hardly any positive characters. Numerous explanations have been adduced by previous workers in this field to account for the profound changes apparently produced in staphylococci by the action of penicillin: the various considerations outlined above, however, taken together with the failure of Paine & Daniel (1959), Briggs *et al.* (1959) and ourselves to repeat completely the work of Briggs *et al.* (1957) lead us to believe that the simplest and most likely explanation is that of contamination. If this is the case, many of the observations that have been made on the metabolism of such penicillin-resistant organisms are fallacious.

It must be admitted, however, that we are unable to explain the findings of Briggs *et al.* (1957). In spite of frequent consultations with Miss Briggs and Dr Gladstone we could not point to any flaw in technique sufficient to account for the frequency with which rods appeared in their staphylococcal cultures while the controls remained negative, nor for the 'reversion' phenomena. Moreover, it is not entirely clear why the penicillin-resistant strains reported by different workers have a general resemblance to each other and to the genera *Achromobacter* or *Alcaligenes*; the latter are common enough in the environment of the laboratory and the cold-room, but contamination by other equally common species might have been expected. In this connexion the resistant Gram-positive coccus of Kawamata & Shigeto (1955) may have been a species of micrococcus unrelated to the 'parent staphylococcus' since their criteria for identification were inadequate, and both positive and negative differences in fermentative capacities were shown. Furthermore, certain differences exist between the various Gram-negative organisms described. Our strain fairly closely resembled G-11, but did not utilize citrate; the slowly growing G-C appeared very different indeed. The organism of Klimek *et al.* (1948) fermented glucose.

In conclusion, while it cannot be dogmatically stated that this form of transformation does not exist, we feel that the onus of proof still remains to show that staphylococci can ever give rise to Gram-negative rods under the conditions reported. It is our belief that contamination is the entire explanation of these phenomena.

It is with great pleasure that we accord our appreciation for the generous help given by Dr G. P. Gladstone and Miss S. Briggs, in providing cultures and enabling us to replicate the minutiae of their technique.

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The Characterization of *Pseudomonas fluorescens*

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SUMMARY: A study of phytopathogenic pseudomonads was begun, but it was found that they could not easily be differentiated from the commonly occurring soil- and water-inhabiting fluorescent pseudomonads. A collection of 169 isolates, including 24 named cultures from various collections, was studied. Both old and new kinds of diagnostic characters, cytological, physiological and biochemical, were investigated under standard conditions. Recordings were made at frequent intervals during a 28-day incubation period. Each character was investigated at least three times during a period of at least five years in order to assess its stability.

A clear differentiation between *Pseudomonas hydrophila* (NCTC 7810) and *P. icthyosmia* (NCTC 8049) and the rest of the collection was apparent; these two isolates belong to the genus *Aeromonas* Kluyver & van Niel. The use of the vibriostatic pteridine derivative O/129 revealed a close relationship between them and *Vibrio icthyodermis* PL 1.

Of the 169 isolates (including at least 20 named species) 165 had 26 common definable and stable characters; these are offered for an extended definition of the genus *Pseudomonas*. A further 43 characteristics were not common to all 165 isolates. No two of these characters were correlated. One isolate had 42/43 positive characters and one had only 4, and there was a very great range of variation. Among 58 selected isolates there were 21 small groups of identical isolates, but the largest number in any group was 5; the remaining 107 isolates each had a different combination of the 43 characters. Even 10 fluorescent isolates, originally obtained from a single plating of 1 loopful of river water, showed 9 different combinations of characters.

A small group of 5 isolates appeared to conform with *Pseudomonas aeruginosa* as defined by Haynes (1951) and Gaby (1955) but many fluorescens-type isolates were closely related when considered on the basis of all the characters investigated. Several of the named phytophathogens showed a close relationship, or apparent identity, with soil and water isolates; many phytophathogens, however, had a conspicuous number of negative characters, suggesting loss of adaptability in a more selective environment.

The 134 soil- and water-type isolates had 11 positive characters, and 9 others almost always positive. On this basis, and adopting the view that all these characters merit equal emphasis (see Tanner, 1918; Sneath, 1957*a*, *b*) it is thought that further divisions into subgroups (species?) are not justified, and that these characters should be used to describe accurately *P. fluorescens* Migula 1894. A definition of this species is suggested.

In spite of the common occurrence of pseudomonads and the large volume of literature on members of this group, their identification and classification is not easy. The potential animal pathogen *Pseudomonas aeruginosa* has been most studied and many now believe that this species, whether forming pyocyanine or not, may be fairly easily distinguished (Haynes, 1951; Gaby & Free, 1953; Gaby, 1955; Gaby & Hadley, 1957). Similarly, increasing knowledge of other economically important pseudomonads, i.e. the phytopathogens, is facilitating their characterization and resulting in more satisfactory identification and classification of a reduced number of species (Elliott, 1951; Dowson, 1957).

However, many studies of the more ecologically specialized pseudomonads seem to have been hampered by the lack of a detailed characterization, by modern standardized methods, of the commonly occurring, saprophytic, (usually) fluorescent, pseudomonads. Model examples of such studies are those of St John-Brooks, Nain & Rhodes (1925) and Clara (1934), who compared green-fluorescent phytopathogens and saprophytes. Clara gave a detailed description of his saprophytic *Pseudomonas fluorescens* (Flügge) Migula 1900, and commented that it would be interesting to know whether the specific characteristics applied to all other green-fluorescent saprophytes. Seleen & Stark (1943) carefully investigated 199 isolates of non-specialized fluorescent pseudomonads and divided their collection into 14 groups on the basis of various physiological characters. Their *P. aeruginosa* group was distinctive, but they admitted that the separation of the other groups was often arbitrary or based on slight differences. They did not find any group which conformed with the description of *P. fluorescens* in *Bergey's Manual* (presumably the 5th ed., 1939). The description of *P. fluorescens* given in the 7th edition (1957) of *Bergey's Manual* has not been revised as compared with that in the 6th edition (1948), and many other soil- and water-inhabiting species are still poorly characterized. In some cases the differentiating criteria are unstable, e.g. pigment production and the reactions of cultures in milk or gelatin media (Rhodes, 1957). The use of the terms '*Pseudomonas* sp.' or '*Pseudomonas fluorescens* species-group' (the latter term introduced by Stanier, 1947, but nowhere defined in detail) is another indication of the need for more precise descriptions and definitions. The results of a study of 169 pseudomonad isolates over a period of seven years, by a wide range of old and new criteria are now presented.

METHODS

Samples of natural waters, soil, sewage, milk, diseased plants, contaminated human blood, etc. were streaked on yeast extract + peptone agar (YE agar) of the following composition (% w/v): Oxoid bacteriological yeast extract, 0·3; peptone (Evans), 1·0; NaCl, 0·5; agar, 2·0; adjusted to pH 7·2–7·4 with NaOH. After incubation for 3 days at 25° colonies which were surrounded by the characteristic green-yellow water-soluble fluorescin were picked off into yeast extract broth (YE broth; i.e. the above medium minus agar). Altogether 145 single-colony isolates were purified by alternate subcultivation of single colonies into YE broth and streaking on YE agar, the two processes being repeated at least five times. Nineteen cultures of phytopathogenic *Pseudomonas* spp. were kindly supplied by Dr W. J. Dowson (Cambridge) and 5 named cultures were obtained from the National Collection of Type Cultures, (NCTC). Table 1 gives details of the isolates used.

The purified isolates were then preserved as 24 hr. cultures (except in the case of five slowly growing phytopathogens which required 48 hr. incubation) on YE agar slopes covered completely with mineral oil (Rhodes, 1957). For the cytological, cultural and biochemical investigations now to be described, a standard inoculum consisting of one 4 mm. loopful of a 24 hr. YE broth (48 hr.

Table 1. *Sources of* Pseudomonas *isolates*

Isolate number	Source and date of isolation (in parentheses)
3	Freshwater algal tank, Botany Dept., Reading University (12. iii. 51)
4/2, 4/3	Puddle, N.I.R.D. Shinfield, Reading (12. iii. 51)
5/1, 5/2, 5/3, 5/4, 5/5, 5/8,	Ornamental garden pond, Warwick Road, Reading (12. iii. 51)
7/3, 7/6	Whiteknight's Park, Reading (17. iii. 51)
8/1 to 8/16	Contaminated human serum, Lister Institute, Elstree (3. iv. 51)
9/1 to 9/4	Large ornamental garden pond, Warwick Road, Reading (11. iv. 51)
10/1, 10/3, 10/4	Freshwater mollusc tank, Zoology Dept., Reading University (11. iv. 51)
12/1, 12/3 to 12/6	Flooded meadow, Whiteknights Park, Reading (11. iv. 51)
13/1 to 13/4	Pond, Shinfield Road, Reading (11. iv. 51)
14/1, 14/2	Ditch, Shinfield Road, Reading (11. iv. 51)
15/1, 15/3 to 15/6	Pond, Whitley Wood Lane, Reading (11. iv. 51)
16/1, 16/2	Ditch, Whitley Wood Lane, Reading (11. iv. 51)
18/1, 18/3	Brown spot lesions on *Primula* sp. leaves; Warwick Road garden, Reading (11. iv. 51)
19	Whiteknights Park Lake, Reading (1949)
20	Microbiology Dept. Stock culture B17; (1949)
21	Septic appendix; Dr T. Richards (1950)
22/1 to 22/7	Raw TT milk, Reading (27. iv. 51)
23/1, 23/3, 23/4, 23/5	Shot-holed lesions *Ilex* sp. leaves, Reading University (1. v. 51)
24/1, 24/2	Calf faeces sample, Royal Veterinary College (10. v. 51)
25/1, 25/2	Aerial laboratory contaminant, Royal Veterinary College (10. v. 51)
26/1 to 26/6	Raw milk sample, Reading (17. v. 51)
27/1 to 27/7	Filter-bed sludge, Reading town waterworks (17. v. 51)
28/3 to 28/6	Pre-filter tanks, Reading town waterworks (17. v. 51)
30	River Kennet (25. v. 51)
31/1 to 31/3	Rotting carrots, Reading (1. vi. 51)
32	Rotting cucumber, Reading; (14. vi. 51)
Isolate number	**Source and date of isolation or receipt (in parentheses)**
34	Rotting cucumber, Reading (20. vi. 51)
35/1 to 35/3	Pond, Mapledurham, Reading (9. vii. 51)
36/1 to 36/4	Pond, Forbury Gardens, Reading (9. vii. 51)
38	Stream, Pinge Wood, Reading (17. vii. 51)
39	Stream (2), Pinge Wood, Reading (17. vii. 51)
40/1 to 40/3	Pond, Pinge Wood, Reading (17. vii. 51)
41	Gravel pit, Burghfield, Reading (17. vii. 51
42/1 to 42/3	River Loddon, Reading (17. vii. 51)
43/1 to 43/4	Ditch, N.I.R.D. Reading (27. v. 51)
45	Tap water from vase containing *Lathyrum odoratum* (25. vii. 51)
46	Rotting carrot, Reading (4. ix. 51)
47/1 to 47/5	Tap water from vase containing *Colchicum* sp. (1. x. 51)
49	Soil, Microbiology Dept. garden, Reading (3. xi. 51)
50	Tap water containing *Ilex* sp. and *Artemis* sp. (13. xi. 51)
51	Aerial contaminant, Microbiology Dept., Reading (15. xi. 51)
52/1 to 52/10	River Kennet, Reading (13. xi. 51)
53/1, 53/2	Stream, Reading (14. xii. 51)
54	River Thames, Reading (14. xii. 51)
55	Puddle, University grounds, Reading (14. xii. 51)
56/1 to 56/4	Soil inoculated into chemically defined inositol medium (14. xii. 51)
d52	Dr W. J. Dowson (WJD) *P. medicaginis* var. *phaseolicola* (b52) (1. xi. 51)
d53	WJD. *P. viridiflava* (53) (1. xi. 51)
d64	WJD. *P. savastanoi* (64) (1. xi. 51)
d69	WJD. *P. tolaasii* (9) (1. xi. 51)
d72	WJD. *P. delphini* (72) (1. xi. 51)
d73	WJD. *P. mori* (73) (1. xi. 51)
d133	WJD. *P. primuli* (133) (1. xi. 51)
d152	WJD. *P. viridilivida* (152) (1. xi. 51)
d221	WJD. *P. tabaci* (221) (1. xi. 51)
d236	WJD. *P. lachrymans* (236) (1. xi. 51)
d244	WJD. *P. barkeri* (244) (1. xi. 51)
d248	WJD. *P. atrofaciens* (248) (1. xi. 51)

Table 1 (*cont.*)

Isolate number	Source and date of isolation (in parentheses)	Isolate number	Source and date of isolation or receipt (in parentheses)
d251	WJD. *P. cruciviae* (251) (1. xi. 51)	5940	NCTC. *P. fluorescens* (5940) (1. xi. 51)
d260	WJD. *P. aptata* (260) (1. xi. 51)	7357	NCTC. *P. chlororaphis* (7357) (1. xi. 51)
d263	WJD. *P. angulata* (263) (1. xi. 51)	7810	NCTC. *P. hydrophila* (7810) (5. iv. 53)
d270	WJD. *P. mors-prunorum* (270) (1. xi. 51)	8049	NCTC. *P. icthyosmia* (8049) (5. iv. 53)
d281	WJD. *P. syringae* (281) (1. xi. 51)	KB 1	Dr M. Kogut, Sheffield strain 4/4, *Pseudomonas* sp. (3. xii. 53)
d289	WJD. *P. marginalis* (289) (1. xi. 51)		
d300	WJD. *P. pisi* (300) (1. xi. 51)		
3246	NCTC. *P. syncyanea* (3246)		

culture for 5 phytopathogens) was always used. Stock 'bench broth' cultures were derived from the oil-preserved slope cultures at 6-monthly intervals, and these 'bench broths' were used to supply the 24 hr. YE broth inocula when necessary.

All cultures when being characterized were incubated aerobically at 25° for 28 days unless otherwise stated, and detailed recordings were made after 1, 2, 3, 7, 14, 21 and 28 days of incubation. In order to assess the stability of the various properties, each character was investigated at least 3 times during a period of at least 5 years.

Cytological studies

Morphology and Gram reaction. Air dried films of 24 hr. YE broth cultures were stained by the method of Gram as modified by Hucker (*Manual of Methods*, 1957, p. 16). Measurements of size were made by using a calibrated eye-piece graticule. Shape and size and the Gram reaction were also noted for organisms from 24 hr. YE agar cultures.

Nuclear staining reaction. Wet films of young (6–8 hr.) YE agar cultures, of 10 isolates only, were fixed in OsO_4 vapour for 1–2 min., hydrolysed in N-HCl for various lengths of time (5–15 min.), stained with dilute Giemsa solution (Gurr's Michrome) at 37° for 30 min. and examined microscopically as wet mounts in dilute Giemsa solution under a coverslip.

Flagella staining. Three methods were investigated. The first was the modification of Morton's Night Blue method as described by Dowson (1949, p. 57), the second used a similar mordant (tannic acid + potash alum) but the dye was basic fuchsin (Leifson, 1951); the third method, which was found to be best, was a modification of Fontana's silver-plating technique (Rhodes, 1958).

Slime and capsule formation. Abundant slime and capsule formation was a striking feature seen in many of the silver-plated preparations made from 24 hr. YE agar cultures and stained to show flagella. Therefore methods recommended for the demonstration of capsules and exocellular slime (Klieneberger-Nobel, 1948, 1950; Novelli, 1953: methods of Leifson (p. 27) and of Anthony (p. 29), *Manual of Microbiological Methods*, 1957). From preliminary experiments it was apparent that the age of the culture was of the utmost importance, because obvious macroscopical mucoidness often appeared, disappeared and then reappeared on ageing. Therefore incubation for 7 days at 25° was chosen as the standard age of culture for cytological examination; to standardize conditions still further, 7-day cultures in Koser's citrate medium (Koser, 1923) were also examined for slime and capsule production by Novelli's Alcian Blue staining method and by the silver-plating method.

Growth characters

(1) *Growth with complex nutrient media*

The appearance of growth in YE broth, used throughout for rapid propagation of pseudomonads, differed with individual isolates. When YE agar was used for the preparation of shake, slope and streak-plate cultures, there were some differences between strains.

(*a*) *Anaerobic growth.* YE broth + 1·0 % (w/v) glucose cultures were incubated for 7 days in a McIntosh and Fildes's jar containing a mixture of 95 %(v/v) H_2 + 5 % (v/v) CO_2. (See also section on nitrate utilization.)

(*b*) *Effect of temperature.* The ability of isolates to grow at 5°, 12–15°, 22°, 30°, 37° and 42° (in water baths; ± 0·5°) in 5 ml. YE broth was examined.

(*c*) *Effect of the initial* pH *value of the medium.* Five ml. volumes of YE broth were adjusted to pH 7·0, 6·0, 5·5, 5·0, 4·5 and 4·0 with N-HCl and used for growth tests in the usual way.

(*d*) *Effect of* NaCl *concentration.* Five ml. volumes of YE broth containing 6·5, 5·0 and 3·0 (%, w/v) NaCl were inoculated and incubated.

(*e*) *Effect of bile salt.* The effect of 0·5 % (w/v) sodium taurocholate (B.P.C. Evans) on the growth of isolates in 5 ml. YE broth was recorded.

(*f*) *Resistance to the pteridine derivative* O/129. Shewan, Hodgkiss & Liston (1954) recommended the use of the vibriostatic compound O/129 (2:4-diamino-6:7-di-*iso*propylpteridine) to differentiate *Vibrio* from *Pseudomonas*, particularly the non-chromogenic pseudomonads. The compound (kindly supplied by Dr H. O. J. Collier) was tested by placing filter-paper strips soaked in a saturated aqueous solution of compound O/129 (sterilized by filtration through a sintered-glass filter) across streak inocula of the individual isolates on YE agar plates. Five *Vibrio* spp. cultures (from NCTC) and one *V. icthyodermis* kindly given by Mr Hodgkiss were used as controls.

(*g*) *Growth on potato slopes.* Potato slopes prepared according to the method described by Mackie & McCartney (1953) were inoculated and incubated.

(2) *Growth with chemically defined media*

(*a*) *Growth in Koser's citrate medium.* The ability of all isolates to grow in 5 ml. Koser's citrate medium (Koser, 1923) from a straight-wire inoculum of a 24 hr. YE broth culture was tested. Falsely positive results were detected by a straight-wire subculture into a second tube of Koser's medium from all the primary cultures which showed growth; further serial subcultures were carried out when necessary.

(*b*) *Utilization of other organic acids as sole added carbon sources.* Since all except 3 isolates grew well in Koser's citrate medium this suggested that ammonium-N was a suitable source of nitrogen for the growth of pseudomonads (cf. Clara, 1934; Seleen & Stark, 1943). Therefore the basal ammonium + inorganic salts medium of Koser, and also the basal ammonium + inorganic salts medium recommended by Dowson (1949), were used to investigate the utilization of the following organic acids as carbon source added to the basal medium without citrate at the indicated concentrations (%, w/v); Na formate, 0·1; Na acetate, 0·2; lactic acid, 1·0; oxalic acid, 0·1; Na succinate, 1·0; l-malic acid, 1·0, tartaric acid, 1·0; citric acid, 1·0. These media were adjusted to pH 7·0–7·2 with NaOH; 5 ml. volumes were sterilized by autoclaving (10 lb./sq. in. for 10 min.). Inoculations were made with a straight wire from 24 hr. YE broth cultures. Subcultures were made when necessary.

(*c*) *Utilization of carbohydrates as sole added carbon sources.* The utilization of the following compounds was investigated: D-glucose, D-galactose, D-fructose, D-arabinose, L-arabinose, D-xylose, L-rhamnose, sucrose, maltose, lactose, trehalose, cellobiose, raffinose, dextrin, starch, cellulose (Whatman's no. 1 filter-paper strips), inulin, salicin, aesculin, sorbitol, mannitol, dulcitol, inositol. In general these carbon substrates were incorporated at 1·0 % (w/v) in Dowson's (1949) ammonium + inorganic salts basal medium. Bromocresol purple (1 ml. of a 1·6 % (w/v) ethanolic solution/l. medium) was added as an indicator. Five ml. volumes of the media were at first sterilized by steaming for 1 hr. on each of 3 successive days; later it was sometimes found necessary to add the separately sterilized sugar solutions to the sterile basal medium because of the formation of toxic products when the complete medium was heat sterilized. The standard inoculum of one 4 mm. loopful was retained because it had been shown that the use of this larger inoculum (as compared with the straight wire inoculum) did not give rise to falsely positive results. Control basal media not containing an added carbon source were also inoculated. The development of turbidity, acidity and gas was recorded.

Note i. The aesculin medium did not contain acid indicator, and the cultures were tested for the presence of the aglycone aesculetin after incubation for 14 days; on adding 2 drops of 5·0 % (w/v) $FeCl_3$ the cultures turned densely black when aesculetin was present. This test was not fully satisfactory because a few isolates grew slightly in the medium and the aesculetin test was doubtfully positive. Therefore Sneath's (1956*a*) recommended methods involving the use of peptone + aesculin agar or broth and the microtest of Cowan (1953) were used to confirm aesculin hydrolysis.

Note ii. The growth test above was considered to be suitable for the detection of starch utilization, but diastatic activity was also investigated by more traditional methods. Streak cultures were prepared on 1·0, 0·5 and 0·1 %, (w/v) starch + YE agar, and the plates flooded with Gram's iodine solution after incubation for 7 days.

(*d*) *Aerobic versus anaerobic glucose utilization.* Ten ml. volumes of the basal peptone agar recommended by Hugh & Leifson (1953) were used; glucose solution sterilized by filtration (sintered-glass) was added to make 1·0 % (w/v). Stab inoculations were made in duplicate cultures. One set of cultures was incubated in the usual way and the duplicates were sealed with a 1 cm. layer of paraffin wax + a 1 cm. layer of mineral oil before incubation.

(*e*) *Gluconate test.* The defined medium of Koser (1923), but with the citric acid replaced by 0·5% (w/v) gluconic acid and neutralized with KOH, was used. Five ml. volumes were inoculated, and after incubation for 7 days 1 ml. Benedict's qualitative reagent for glucose was added. Then, after standing 10 min. at room temperature, the cultures were heated at 100° for 10 min., and examined for reduction (Sneath, 1956*a*).

(*f*) *Voges-Proskauer* (*V-P*) *and methyl red* (*MR*) *tests.* Fouad & Richards (1953) recommended a glucose + ammonium + inorganic salts medium for examining the V-P and MR reactions by members of the Enterobacteriaceae; 7 ml. volumes of this medium were used here. The V-P and MR tests were carried out on 1 ml. samples of the cultures after 5 and 7 days of incubation. The O'Meara (1931) method of testing for acetoin was used.

(*g*) *Utilization of miscellaneous carbon compounds.* The following compounds were tested for their ability to support growth when added as carbon sources to the basal medium of Dowson (1949; %, w/v): ethanol, 0·5; glycerol, 1·0; phenol, 0·1; *m*-cresol, 0·1; naphthalene was used as whole medium saturated with solid recrystallized naphthalene. Five ml. volumes of medium were used for the growth test.

(*h*) *Utilization of ammonium-N.* (See Koser's citrate test, (*a*), above). Dowson's (1949) basal medium + 1·0% (w/v) glucose, galactose or citrate was also used. For every isolate a control tube of basal medium not containing added N-source (KH_2PO_4 replacing $NH_4H_2PO_4$) was similarly inoculated and incubated.

(*i*) *Utilization of nitrate-N.* The ability of nitrate-N to support growth was investigated by using the following medium (%, w/v); K_2HPO_4, 0·1; KCl, 0·02; $MgSO_4 . 7H_2O$, 0·02; KNO_3, 0·1; galactose, 1·0; adjusted to pH 7·0 with N-NaOH. Duplicate cultures were incubated both aerobically and anaerobically for 7 days, and then examined for growth, for nitrite (Griess–Ilosvay reagents), for ammonia (Nessler's reagent) and visible gaseous products (accumulated in a Durham tube). All negative nitrite tests were checked for the presence of residual nitrate by the zinc dust method (ZoBell, 1932). Any reduction of nitrate after 7 days of aerobic incubation in conventional 0·1% (w/v) KNO_3 + peptone water medium was also similarly investigated in order to compare the properties of the organisms in the present collection with published descriptions of named pseudomonads. Microtests for the detection of nitrate-reducing ability by heavy suspensions were made by the method recommended by Brough (1950).

(*j*) *Utilization of nitrite-N.* Four methods were used. (1) Tests for growth and nitrite disappearance were made with the same glucose + inorganic salts basal medium as above but with the KNO_3 replaced by $NaNO_2$. Because of the possible toxicity of nitrite, three concentrations (0·1, 0·05, 0·01, %, w/v), were used. After 7 days of incubation nitrite was tested for by means of the Griess–Ilosvay reagents. (2) Koser's citrate medium + 0·0005% (w/v) $NaNO_2$ was similarly used for growth and nitrite-disappearance tests. (3) Cultures in YE broth + 0·0005% (w/v) $NaNO_2$ + 0·25% (w/v) agar were also tested for nitrite disappearance after 1 and 5 days of incubation (Sneath, 1956*a*).

(4) The Brough (1950) micromethod was adapted for testing for the disappearance of nitrite after incubating heavy suspensions in YE broth $+0{\cdot}005\,\%$ (w/v) $NaNO_2$.

(*k*) *Utilization of urea.* Christensen's (1946) urea+peptone agar method was used to detect urea-decomposing isolates; this medium was not entirely suitable for pseudomonads because of simultaneous alkali formation from the peptone. Therefore a chemically defined medium with 2·0 % (w/v) urea (Seitz-filtered) as sole added nitrogen source was also used; the basal medium contained phenol red and it was essentially that of Dowson (1949) except that the carbon source (galactose) was at 0·1 % (w/v), to prevent acid production masking or inhibiting the urease action.

Biochemical tests

(*a*) *Catalase production.* A loopful of solid growth taken from a 24 hr. YE agar slope was removed into a drop of '10 vol.' hydrogen peroxide solution and examined for the production of gas bubbles.

(*b*) *Ammonia production from peptone.* Peptone water (YE broth minus yeast extract) in 5 ml. volumes was used. After incubation for 7 days the pH value of each culture was measured electrometrically, and ammonia was tested for by adding 2 drops of Nessler's reagent.

(*c*) *Haemolysis.* Streak cultures on 5 % (v/v) horse blood YE agar plates were examined for haemolysed zones.

(*d*) *Reaction on egg-yolk medium.* The appearance of the isolates when grown as surface streaks on egg-yolk plates (Knight & Proom, 1950) was recorded.

(*e*) *Gelatin hydrolysis.* Stab inoculations into nutrient gelatin (YE broth + 12·0 %, w/v, Oxoid gelatin) were made. Cultures were incubated at 22° and examined at intervals, the shape and approximate amount of liquefaction, when present, being noted. The possibility that the peptone might be preferentially used was considered, and so a similar medium without peptone was used for comparison. The streak plate method of Frazier (1926) for detecting gelatin hydrolysis was also used, and the width of the hydrolysed zone was measured after 7 days of incubation.

(*f*) *Liquefaction of pectate gel.* The method recommended by Dowson (1949) was used. Stab inoculations were made into a gel of calcium pectate, prepared by layering a solution of sodium pectate (A.S.P. Chemical Co. Ltd.) over calcium chloride agar. Cultures were examined for growth and liquefaction.

(*g*) *Action on milk.* Seven-ml. volumes of litmus milk and of methylene-blue milk (methylene blue, 1/20,000) were inoculated and examined for colour changes, clotting, casein digestion and dye reduction. Because of the difficulty of detecting milk-protein digestion in strongly alkaline liquid cultures, the method involving spot inoculation of cultures on to skim-milk agar plates (skim milk, 20 % (v/v); washed agar, 2·0 % (w/v); adjusted to pH 7·2) was also used. After 7 days of incubation the plates were flooded with $HgCl_2$ solution (Frazier, 1926), and any clear zones of hydrolysis measured.

(*h*) *Indole production.* Five ml. volumes of peptone (Evans) water cultures were incubated for 7 days, and then shaken with 1 ml. ether to extract indole which was detected by means of Ehrlich's reagents. The oxalic acid test paper method was used with *Pseudomonas aeruginosa* (to test for false positive results due to conversion of pyocyanine to its red salt in acid solution; Sandiford, 1937). Ability to produce indole from tryptophan was similarly investigated by using the Dowson (1949) galactose + ammonium + inorganic salts medium + 0·01 % (w/v) tryptophan.

(*i*) *Production of hydrogen sulphide.* All isolates were first tested for their ability to produce H_2S from Kligler's iron agar (Oxoid) medium. A few isolates gave a positive reaction; the H_2S might have arisen from S-containing amino acids or sodium thiosulphate in the medium, or from both. Therefore two chemically defined media were devised, one with cystine (0·01 %, w/v) added to the Dowson-type ammonium + galactose + inorganic salts medium, the other with 1·0 % (w/v) sodium thiosulphate added to the same basal medium. H_2S was detected by sterile lead acetate papers held in place by the cotton-wool plugs of the cultures. After 7 days of incubation the cultures were also tested for dissolved sulphides by the addition of lead acetate solution. Because some results were inconclusive, a microtest was also used; heavy suspensions of organisms grown on YE agar for 24 hr. were suspended in 1 ml. of each of the two chemically defined media and incubated at 37° for 24 hr. with lead acetate papers held in place by cotton-wool plugs. No carbon source was added to the thiosulphate-containing suspending medium because of the report by Leathen & Braley (1955) of the chemical decomposition of thiosulphate to sulphate, hydrogen sulphide and sulphur at acid pH values.

(*j*) *Hydrolysis of tributyrin.* A stable emulsion of 1·0 % (v/v) tributyrin in YE agar was prepared by blending in a hand emulsifier; streak cultures were made on poured plates of this medium. Clear zones of hydrolysis were measured after different incubation periods.

(*k*) *Hydrolysis of olive oil.* Because the triglycerides of olive oil are composed of higher molecular-weight fatty acids than is tributyrin it was thought that this might affect the ease with which they were hydrolysed by pseudomonad enzymes. To encourage potential ability to hydrolyse olive oil, this was incorporated as the major added carbon source in the following medium (%, w/v): $NH_4H_2PO_4$, 0·1; KCl, 0·02; $MgSO_4.7H_2O$, 0·02; yeast extract (Oxoid), 0·3; agar, 2·0; olive oil, 5·0: adjusted to pH 7·8 with NaOH. The olive oil contained the base of Night Blue to a final concentration in the medium of 1/15,000. This method was adapted from that of Jones & Richards (1952). Streak plate cultures were examined for growth and the production of a blue salt (formed by the reaction of the liberated fatty acids with the weak base of the dye). Controls of all isolates were grown on plates of similar medium but without olive oil.

(*l*) *Hydrolysis of margarine.* The test method was similar to that of (*k*) and again depended upon staining the margarine with the base of Night Blue and then incorporating 5·0 % (v/v) of it into YE agar.

(*m*) *Hydrolysis of Tween* 80. The use of the water-soluble Tweens for easy detection of lipolytic activity by micro-organisms including *Pseudomonas aeruginosa* was described by Sierra (1957*a*). His technique, using 1·0 % (v/v) Tween 80 (polyoxyethylene sorbitan mono-oleate) in calcium chloride +

peptone agar, was adopted; parallel cultures on tributyrin + YE agar were simultaneously inoculated. After incubation the Tween cultures were examined for the presence of opaque haloes, due to the formation of conspicuous precipitates of calcium oleate round the zones of growth.

RESULTS

Morphology and Gram reaction. When YE broth and YE agar cultures (aged 18–24 hr.) were examined by the Gram method, only Gram-negative rods were found. The usual width was 0·5–0·6μ; a width of 0·7μ was recorded for 15 isolates. Average cell length was 1·5–2·0μ; with a range from 1·0–3·0μ. With increasing age a greater proportion of the rods were of maximum length, but old cultures (14 days) showed only Gram-negative granules and debris. A few peculiarities were: (i) in some preparations all the rods were only faintly stained (controls on the same slides were normal); (ii) the arrangement of the rods was usually random from broth cultures, but from agar cultures the rods were often arranged in a reticulum; this mesh-work was fine or coarse, and the appearance suggested that a slime was holding the rods together, although no such material was demonstrable by the Gram method; (iii) other rod arrangements noted were, longitudinal pairs, short or long chains of rods, long filaments (apparently acellular). All these minor variations were not constant for a given isolate except for chain formation by three isolates, a faint Gram-negative reaction by two isolates and filament formation by four isolates.

Nuclear staining reaction. In spite of many attempts with different periods of hydrolysis and staining, and with cultures of different ages, very few convincing appearances of chromatinic bodies were obtained, although distinct positively stained bodies were visible in *Bacillus cereus* and *Oidium lactis* controls. In 12 hr. cultures of 7 isolates, small rods with one or two subpolar granules/rod, and longer rods with more granules, were seen. It is felt that the main difficulty is an optical one because of the small size of the rods.

Flagella staining. When young YE broth cultures were examined as hanging-drops, rapid motility was usually obvious. Of the flagella-staining methods tried, the silver-plating technique (Rhodes, 1958) was found to be superior in every way. Rhodes (1958) did not find any non-flagellate isolate of *Pseudomonas*, nor was any loss of flagella during maintenance of stock cultures on laboratory media seen; the number of flagella/rod, amphitrichy, and degree of flagellar curvature were found to be variable characters of little value for strain identification. For every isolate polar flagella were demonstrated, 1–3 flagella/rod being characteristic of *Pseudomonas*, but numbers up to 10 were common.

Slime and capsule formation. Abundant slime formation was a feature of many preparations stained by the silver-plating method to show flagella (Rhodes, 1958). The amount of exocellular slime varied from abundant to very slight, but it was rare for a pseudomonad not to produce any slime, whatever the age of the culture (12 hr.–28 days). The results of Alcian Blue staining and the silver-plating method were in good agreement. Klieneberger–

Nobel's (1950) staining method for detecting exocellular slime was followed using 7-day YE broth and Koser's citrate medium cultures; the results were not similar (quantitatively or qualitatively) in the two media; but only five isolates showed complete absence of slime. Considerable exocellular slime production was characteristic for 164 of 169 *Pseudomonas* isolates investigated. (See Eagon, 1956, for conditions affecting slime production by *P. fluorescens*; Fuchs, 1956, concerning levan synthesis from sucrose by three strains of non-phytopathogenic *P. fluorescens*.)

In some isolates distinct capsules were apparent round a few organisms in a preparation, but this character did not appear to be constant or stable because even in one microscope field some rods were capsulated while others were not. Usually the capsules were not conspicuous; the typical width was 0·4μ or less. Table 2 shows that both the medium in which the organism was grown and the age of the culture influenced capsule formation. This cytological character appears to be of little use for diagnostic purposes.

Table 2. *Influence of medium and age of cultures on capsulation in 169 isolates of* Pseudomonas

Each isolate was examined by the Alcian Blue staining method (Novelli, 1953) and the silver-plating method (Rhodes, 1958).

Cultural conditions	Isolates which showed some capsules (%)
1 day on yeast-extract agar	58
7 days in yeast-extract broth	42
7 days in Koser's citrate medium	27

Growth characters

(1) *Growth with complex media*

It is not possible to describe here the cultural characters of all isolates when they were grown in YE broth or on YE agar slopes, in stabs, shakes or surface streak-plate cultures. Only the usual characteristics of the growth in each type of culture are given below. The observations were made after inoculation with a standardized inoculum from a 24 hr. culture on YE broth, during incubation for 28 days at 25°.

YE broth. At 1 day: slight–moderate homogeneous turbidity. No deposit. No pellicle. No pigment. No odour. Slight frothing of medium on shaking.

At 2 days: moderate–dense turbidity. Slight easily dispersed white deposit. Thin pellicle and ring. Slight green fluorescent pigment, more noticeable at the surface. Slight sweet fishy odour. Slight frothing and a characteristic cloudy, swirling effect on shaking, becoming homogeneous but for broken pellicle.

At 3 days as after 2 days but pigment more intense.

At 7 days: slight turbidity. Much whitish deposit, seen to be composed of slimy strands or a dense slimy mass on shaking. Thin pellicle (white). Marked white ring. Green-yellow soluble pigment of maximum intensity. Strong mawkish sweet fishy odour. Considerable frothing of medium on shaking.

At 14 days: as at 7 days but turbidity very slight and slimy deposit abundant. More marked frothing on shaking. Pigmentation more yellow-orange; pH value 8·3–8·7.

At 21 days: no further change.

At 28 days: slight turbidity. Abundant mass of slimy whitish growth at base of tube. Medium orange-brown. Viable.

YE agar slope. At 1 day: thin, spreading, cream-white growth. Transparent by transmitted light. Smooth, or slightly 'beaten metal' surface. Edge entire or crenate. Slight sweet fishy odour. Moderate amount of green-yellow fluorescent pigment in the medium. Butyrous consistency. Easily emulsifiable with water forming a homogeneous white suspension.

At 2 days: good growth, slightly thicker but still transparent by transmitted light. Pigment more pronounced. Marked fishy odour.

At 3 days: no further changes.

At 7 days: as at 2 days but more marked 'beaten metal' surface. Green-yellow pigmentation of medium maximal. Very slight odour. Translucent by transmitted light. Edge slightly crenate. No further changes.

At 14 days: on subsequent incubation the only conspicuous change was a slow darkening of the medium from yellow→orange→brown, more marked indentation of the edge and increasing transparency of the growth as viewed by transmitted light.

At 28 days: still viable.

YE agar stab. At 1 day: cream colony on surface at point of inoculation.

At 2 days: larger surface colony and faint growth along line of stab.

At 3 days: no further changes.

At 7 days: cream surface colony spreading to periphery. Growth along line of stab thicker in upper region, but still slight. Very slight green pigmentation in upper 5 mm. medium only.

At 14 days: no further changes on prolonged incubation.

At 28 days: still viable.

YE agar shake. At 1 day: slight film of whitish growth at surface only.

At 2 days: Moderate amount of white growth at surface only

At 3 days: no further changes.

At 7 days: thick film of cream-white surface growth only, with very slight production of green pigment in upper 5 mm. medium only.

At 14 days: no further changes on prolonged incubation.

At 28 days: still viable.

Colony on YE agar. At 1 day; 0·5 mm. diameter. White-cream coloured. Low convex. Surface smooth and shining. Transparent by transmitted light. Medium slightly coloured due to production of green-yellow fluorescent pigment. Butyrous consistency. Colony easily emulsified with water forming a homogeneous white suspension. Slight sweet fishy odour.

At 2 days: circular 1·5–2·0 mm. diameter. Cream-white colour. Low convex. Surface smooth and shining. Transparent by transmitted light. Edge entire. Moderate production of green-yellow water-soluble fluorescent pigment.

Marked mawkish sweet fishy odour. Butyrous. Easily emulsified with water forming a homogeneous white suspension.

At 3 days: little further change.

At 7 days: circular, 4·0–5·0 mm. diameter. Cream coloured. Flattened, or very low convex contour. Surface smooth and shining. Transparent or translucent by transmitted light. Edge entire. Maximum production of green-yellow water-soluble fluorescent pigment. Mawkish sweet fishy odour. Butyrous. Easily emulsified with water giving a homogeneous white suspension.

At 14 days: as at 7 days except for increased flattening, and the differentiation of an even, narrow, very thin flat margin *c.* 0·5 mm. wide; surface of 'beaten metal' appearance; pigments in medium turning more yellow–orange.

At 21 days: as at 14 days but differentiated margin more conspicuous; edge becoming crenate or irregularly lobed; transparent by transmitted light and medium pale brown.

At 28 days: approximately circular, cream fawn colour; 6·0–8·0 mm. diameter. Moderately wide, very flat, thin margin with lobed or crenate outline. Whole colony very flat and transparent by transmitted light. Surface may be smooth, but usually 'beaten-metal', and shining. Medium pale brown. Butyrous consistency. Emulsion in water cream-white and homogeneous. Marked unpleasant mawkish sweet fishy odour. Organisms viable.

General comments. Individual isolates showed minor variations such as lack of pellicle formation, slight or intense pigmentation, dull or rough surface appearance and differences in opacity of the growth on solid media. Differences in the colonial appearance of streak-plate cultures were common. No full explanation of the various colonial types encountered is yet possible, although further investigations (cytological, physiological, biochemical) have been carried out. So far it has been found that even the most conspicuous colonial differences are only rarely correlated with any qualitative differences in the diagnostic criteria described in this report (contrast Williamson, 1956, who had colonial variants of *Pseudomonas aeruginosa* which showed differences in mouse virulence and carbohydrate utilization). Others, e.g. Suire (1958), have described the production of spontaneous colonial mutants or dissociants by *Pseudomonas* spp.

(*a*) *Anaerobic growth.* Only two isolates, 7810 (*Pseudomonas hydrophila*) and 8049 (*P. icthyosmia*) grew under strictly anaerobic conditions in glucose YE broth; in this they grew equally well aerobically or anaerobically. The organisms in all cultures were still viable after anaerobic incubation for 7 days, but are regarded as obligate aerobes; this agrees with the views of Seleen & Stark (1943).

(*b*) *Effect of temperature.* Most isolates grew well at all temperatures between 5° and 30° and, with few exceptions, there were no clear-cut temperature optima between 12° and 30°, either on the basis of rate of growth or the final amount of it. Nine isolates only were able to grow well at 37°; these grew also at 42°. Only five isolates failed to grow at 5° (20, 21, 24/1, 24/2 and d73), but all of these except d73 were able to grow at 42°. Thus the published opinions (Seleen & Stark, 1943; Gaby, 1955) concerning temperature and *Pseudomonas* growth were confirmed; the only three known *P. aeruginosa* (pyocyanine-producing) isolates of the collection (20, 24/1, 24/2) did grow at 42°; no pyocyanine was produced by any of these isolates at 12° or 42°. Of the remaining six isolates which grew at 42°, d73 was a peritrichously-flagellate rod; 7810 and 8049 were known to be animal pathogens and facultatively anaerobic polar flagellates of the genus *Aeromonas* (Kluyver & van Niel, 1936). Isolate 5940 was supplied from the NCTC as *P. fluorescens*; 21 was obtained from an inflamed human appendix; it is possible that these last two isolates were apyocyaninogenic strains of *P. aeruginosa*. Isolate 55 seemed unique as a saprophytic pseudomonad able to grow at 42°, but in fact it was no longer typical, for it had early undergone a change to give a slow-growing organism not producing fluorescin and with an unusual pin-point colonial morphology. It is concluded that the growth temperature alone is of limited use for the identification of polarly-flagellate rods because of their normally wide temperature range; and the 42° growth test must be interpreted in conjunction with other characters, as emphasized by Haynes (1951).

(*c*) *Effect of the initial* pH *value of the medium.* All isolates grew well in YE broth with an initial pH value of 7·0 or 6·0, none grew at pH 4·5 or 4·0. Only isolates d64 and d73 failed to grow at pH 5·5 and all except seven isolates grew when the initial pH value of the medium was 5·0. It is noteworthy that nearly all of the aberrant isolates were named phytopathogens.

(*d*) *Effect of* NaCl *concentration.* All isolates grew well in YE broth + 3·0% (w/v) NaCl; with isolate d72 (*Pseudomonas delphini*) only, was growth slow. In 5·0% (w/v) NaCl medium growth of 151 (89%) of the isolates was found, in contrast to the observations of Seleen & Stark (1943) who found that very few pseudomonads grew in their chemically defined asparagine medium + 5·0% (w/v) NaCl. In the 6·5% NaCl + YE broth rapid growth was uncommon but 32 (18·9%) isolates grew after a lag period, often of 7–14 days duration; Seleen & Stark obtained no growth in their 6·5% NaCl medium. Typically in the NaCl + broth cultures a dense slimy mass accumulated at the base of the tube, leaving the supernatant fluid almost clear; other features were the absence of fluorescent pigment (all cultures) and relatively lower salt tolerance of many of the named phytopathogens examined.

(*e*) *Effect of bile salt.* Many isolates (146/169; 86%) grew well in the bile salt medium, but usually incubation for 3 days was necessary before definite turbidity was seen. Four phytopathogens did not grow in the medium and the remaining 19 isolates showed only sparse turbidity after incubation for 28 days. Isolate 7810 (*Pseudomonas hydrophila*) showed a slight acid reaction. An incidental observation of importance in water-testing was that *P. aeruginosa* grew well on MacConkey agar at 37°.

(*f*) *Resistance to the pteridine derivative* O/129. This compound had no effect on the growth of any of the true *Pseudomonas* isolates (i.e. excluding the two *Aeromonas* spp.); unfortunately none of the five NCTC *Vibrio* spp. cultures were affected either. Isolate d73, obtained as *P. mori* but found to be a rod with peritrichous flagella, was the only susceptible organism found; in all tests at least a 4 mm. zone of inhibition of growth was evident. Because of these unpromising results Dr H. O. J. Collier kindly supplied a sample of the more water-soluble phosphate derivative of O/129, and Mr Hodgkiss sent a sensitive culture of *Vibrio icthyodermis* PL1 as a control culture for further experimentation. *V. icthyodermis* was sensitive to the original compound O/129 but a wider zone of inhibition was noted with the phosphate derivative (15 mm. zone compared with 10 mm.); the NCTC *V. alcaligenes* also showed a 7 mm. zone of inhibition. Of the *Pseudomonas* isolates, only 7810 and 8049 (the two Aeromonas isolates) were inhibited by the phosphate derivative; no growth was visible in a zone 5 mm. wide. After a comparative investigation of the three sensitive isolates, *V. icthyodermis* PL1, 7810 (*P. hydrophila*) and 8049 (*P. icthyosmia*), I believe them to be very closely related; whether they are to be regarded as *Vibrio* or *Aeromonas* spp. is a side-issue here. It was concluded that polarly-flagellate, aerobic, oxidative pseudomonads were not inhibited by the vibriostatic pteridine derivatives tested.

(*g*) *Growth on potato slopes.* All isolates except d73 grew on potato slopes. Abundant growth, variously pigmented (cream, fawn, yellow, orange, black) was noted after 2–3 days. The medium was also usually pigmented; the potato tissue turned various shades of brown and fluorescin was produced by most of the cultures. Because different batches of potato gave rise to different types of growth and pigmentation, further details were not useful. No marked maceration of the potato was evident in any culture.

(2) *Growth with chemically-defined media*

(*a*) *Growth in Koser's citrate medium.* All isolates except 25/2, d73 and 7810 grew well in this medium. The three exceptions were anomalous in other respects; isolate 25/2 resembled *Xanthomonas* in many growth and cultural characters; isolate d73 was peritrichously flagellate and isolate 7810 was an *Aeromonas* spp. Therefore this growth test appeared useful as a simple diagnostic criterion for a *Pseudomonas* sp., confirming the findings of Clara (1934), Seleen & Stark (1943), Erikson (1945) and Naylor (1954). The mineral salt composition of this medium was obviously suitable for Pseudomonas growth.

(*b*) *Utilization of other organic acids as sole added carbon sources.* With the described test method, and either the Dowson-type or the Koser-type basal salts media there were four kinds of results: isolates which failed to grow; isolates which produced sparse turbidity after prolonged incubation which was not reproducible on subculture; isolates which slowly developed a sparse turbidity which was subculturable (at least three times); isolates which grew well and rapidly. The results obtained for five organic acids or their salts are given in Table 3; 148 isolates grew with succinate, acetate, lactic acid, formate, 1-malate or citrate acid as the sole added carbon source. These results are similar to those of Seleen & Stark (1943) except that none of their organisms utilized formate; however, they used a 0·3% (w/v) concentration while only 0·1% was used here. The results of some preliminary work concerning these simple growth tests are worth noting. It was found that for acetate or succinate utilization the Koser-type basal salts medium was more favourable for growth than the Dowson-type medium, e.g. in one experiment 100 isolates did not grow in Dowson-type medium + acetate, whereas only 23 isolates did not grow after 14 days of incubation in the Koser-type basal medium + acetate. The concentration of carbon source was also important, but more so in the Dowson-type basal medium and particularly with acetate: e.g. 17/69 isolates did not grow in 1·0 % acetate medium but grew well in the presence of 0·1 or 0·5 %. A further 16 isolates did not grow in 0·5 % acetate but grew in 0·1 % acetate in Dowson-type basal medium. Another concentration effect was also found in the 1·0 % (w/v) lactic acid medium, in which two isolates (18/3, d133) did not grow, but they did grow with 1·0 % ammonium lactate in a Koser-type salts medium.

Table 3. *The utilization of organic acids by* 169 *isolates of Pseudomonas*

The inorganic salts + citrate medium of Koser (1932) was used, and also the same basal medium but with citric acid replaced by (%, w/v): 1·0 sodium succinate; 1·0 lactic acid; 1·0 L-malic acid; 0·2 sodium acetate; 0·1 sodium formate. + indicates growth; − indicates no growth.

Isolate		Carbon source					
		Succinate	Acetate	Lactate	Formate	L-Malate	Citrate
d152	*P. viridilivida*	−	+	+	+	+	+
23/1	From *Ilex* lesions	−	+	+	+	+	+
18/3	From *Primula* lesions	+	+	−*	+	+	+
55	From puddle	−	−	+	+	+	+
d236	*P. lacrymans*	−	+	−	+	+	+
27/3	From water works	+	−	+	−	+	+
d270	*P. mors-prunorum*	−	−	−	+	+	+
d133	*P. primuli*	−	−	−*	+	+	+
d221	*P. tabaci*	−	−	−	+	+	+
d281	*P. syringae*	−	−	−	+	+	+
10/1	From mollusc tank	−	−	+	−	+	+
d248	*P. atrofaciens*	−	−	+	−	+	+
d260	*P. aptata*	+	−	−	−	+	+
7810	*P. hydrophila*	+	+	−	−	+	−
18/2	From *Primula* lesion	−	−	−	−	+	+
d72	*P. delphini*	−	−	−	−	+	+
d64	*P. savastanoi*	−	−	−	−	+	+
d69	*P. tolaasii*	−	−	−	−	+	+
d300	*P. pisi*	−	−	−	+	−	+
25/2	Probably *Xanthomonas*	−	−	−	−	−	−
d73	*P. mori* (peritrichous flagellation)	−	−	−	−	−	−
Remaining 148 isolates		+	+	+	+	+	+

* Grew in 1·0 % ammonium lactate medium

The effect of a growth factor supplement (0·03 %, w/v, Difco yeast extract) to the Dowson-type + acetate or succinate media was investigated; with acetate 34/46 isolates, and with succinate 30/62 isolates apparently grew only when yeast extract was present. But for many of these isolates, substitution with the Koser-type basal salts medium resulted in growth in the absence of yeast extract. This effect was confirmed in the case of formate utilization; yeast extract had no true growth factor effect on formate utilization when the Koser-type basal medium was used.

Further work is necessary concerning tartaric acid utilization because the results were not always repeatable, and the exact chemical (isomeric) nature of the tartaric acid crystals used was not ascertained. At least 44% of the isolates used this acid as a sole added carbon source. The oxidation of the various isomers of tartaric acid by *Pseudomonas fluorescens* was reported on by Martin & Foster (1957) and for a soil *Pseudomonas* sp. by Shilo & Stanier (1957).

In the 0·1% oxalic acid Dowson-type medium 81/169 isolates developed a slight, although definite, turbidity after 3–7 days; there was little increase in opacity on further incubation. Similar growth was, however, obtained after two subcultures into tubes of similar medium. This restricted growth may have been due to an unfavourable basal medium, the limited amount of oxalate, or to carbon impurities in the medium. It was found that all the positive isolates developed a similar slight turbidity in the control medium which did not contain any added carbon source. Because of these results the calcium chloride + potassium oxalate agar method of Jayasuriya (1955) for the direct detection of oxalate-decomposing pseudomonads and vibrios was also used. Although all the isolates grew on this medium none gave any reaction indicative of oxalate decomposition; compare *Pseudomonas oxaliticus* (Khambata & Bhat, 1953).

(*c*) *Utilization of carbohydrates as sole added carbon sources.* The first routine investigation revealed that 40 of the isolates did not grow in the D-glucose medium. The test was repeated five times and altogether 64 isolates did not grow in at least one test. Further investigations with simultaneously replicated cultures, different sizes of inoculum, media in which the glucose was separately sterilized by either steaming, autoclaving or Seitz-filtration, and also with different single colonies of a given isolate as inoculum, were carried out. The results of this work may be summarized as follows. (i) There was often poor agreement of results when quadruplicate series of glucose + salts cultures were inoculated simultaneously from the same 24 hr. YE broth culture. (ii) The size of the inoculum (i.e. straight wire or 4 mm. loop) was only rarely important, but there was a tendency for the larger inoculum to establish itself more frequently. (iii) There was a strong indication that heat-sterilization of the complete medium produced a medium inhibitory to the growth of at least 46% of the isolates; heat-sterilization of the glucose solutions separately, either by steaming or autoclaving, only rarely inhibited growth. Apparently the substance(s) in the inhibitory medium was actively toxic and not merely bacteriostatic, because after 14 days of incubation the ‘no growth’ tubes contained no viable organisms, while controls remained viable for 6 weeks in sterile tap water. Some isolates were more consistently affected by the supposed inhibitory compound(s) than others. This sensitivity was not confined to the known plant pathogens. (iv) Cultures derived from different single colonies of an isolate frequently showed different sensitivities toward the hypothetical inhibitory substance(s) in the steam-sterilized (100°) complete glucose medium; even cultures inoculated from different zones of the same colony showed different results in 21/35 instances. The slime produced by mucoid colonies had no protective effect. Altogether 273 colonies or parts of colonies from 66 isolates were tested in duplicate in the steam-heated complete glucose medium, and in medium to which glucose separately sterilized by Seitz-filtration was added. Of the colonies tested 37·3 % showed a sensitivity towards the supposed inhibitory compounds formed when the complete glucose + ammonium + salts medium was sterilized at 100°. Finally, even when the glucose was sterilized separately seven isolates were unable to use it as a carbon source for growth in the Dowson-type ammonium + salts medium. Apart from isolate d73, all these isolates were named phytopathogenic *Pseudomonas* spp., so that non-utilization of glucose under these conditions seems to be a real property of a few pseudomonads.

Because of the above results, similar 'no growth' phenomena in other carbohydrate-containing media were confirmed with separately sterilized carbohydrate solutions. Mannitol, salicin and sucrose sometimes caused inhibition of growth, but not so often as glucose.

Galactose, fructose, maltose, D- and L-arabinose, lactose and cellobiose did not cause any similar growth inhibition. This extends the reports of sugar-containing media which inhibit the growth of other bacteria; see Lewis (1930) for *Phytomonas* (*Xanthomonas*) *malvacearum*; Stanier (1942) for *Cytophaga*; Jensen (1951) and Parker (1955) for *Azotobacter* (see also van Steveninck, 1958).

The results of 17 out of 23 of the carbohydrate–carbon utilization tests are given in Fig. 1. For the six remaining substrates, dextrin, starch, cellulose, inulin, salicin and dulcitol, it was found that with very few exceptions a certain 82 isolates showed a slight to moderate, but distinct, turbidity. For these same 82 isolates, slight turbidities had also been recorded in the oxalate medium (see above). Furthermore, precisely these 82 isolates showed a similar slight growth in the control medium which contained no added carbon source; possibly growth occurred at the expense of intracellular food reserves or of carbonaceous impurities in the medium. Garvie (1955) reported experiments in which *Pseudomonas fluorescens* grew (to 1–10 million organisms/ml.) and was repeatedly subculturable in a very pure solution of phosphate buffer; it was claimed that there was no significant carry-over of nutrients, and that growth was not supported by dead organisms. A similar very small degree of multiplication (not giving growth visible to the naked eye) of *P. fluorescens* was reported by Englesberg & Stanier (1949) in studies on itaconate-utilizing mutants. Because of these reports, the correlating carbohydrate-utilization tests are recorded as one character only in Fig. 1, in the 'control, minus carbon' column.

Note i. Concerning aesculin utilization. There was finally good agreement in the results with the four methods of test for aesculetin formation. The micromethod was the most rapid; isolates usually gave a definite reaction after incubation for 24 hr. although a few required 3 days. Sneath's agar plate method was preferable to his broth method because of greater rapidity and sharpness of results. There was no correlation between the ability to hydrolyse aesculin and any other character tested. This was disappointing in comparison with its usefulness in the classifying of the closely-related genus *Chromobacterium* (Sneath, 1956*a*).

Note ii. Falsely positive growth results were obtained when the liquid starch medium was used, except in the case of the two *Aeromonas* spp. which both grew rapidly in the medium, producing dense turbidity, acid and gas; they also grew rapidly and were the only isolates to produce acid and gas from dextrin and maltose. By the traditional starch agar plate method, only these two isolates hydrolysed starch significantly. Preliminary experiments showed that the concentration of starch in the YE agar was important (0·1 %, w/v, optimal) and on such iodine-flooded starch cultures about 20 Pseudomonas isolates showed ill-defined reddish zones indicative of erythrodextrin formation. Several more isolates were feebly diastatic, showing signs of hydrolysis only immediately below the areas of growth. There was no agreement between the liquid starch medium results and the feeble reactions on starch agar; it was concluded that the only value of this test in this survey was for the detection of *Aeromonas* spp. Some feeble starch reactions were found also by Clara (1934), but all the 199 isolates of Seleen & Stark (1943) were non-diastatic.

Note iii. Simon (1956) suggested that the fermentation of D-arabinose in peptone-free medium differentiated *Pseudomonas aeruginosa* from *P. fluorescens*. This was not confirmed in the present work because only 24 isolates did not utilize D-arabinose; however after only 48 hr. of incubation many *P. fluorescens*-type isolates showed only sparse growth. L-Arabinose was used more rapidly, but the D-form was not utilized faster by the pyocyanine-producers and at least five fluorescens-type isolates utilized both isomers with equal facility. Palleroni & Doudoroff (1957) found that mutants of *P. saccharophila* were able to oxidize D-arabinose; Lockwood & Nelson (1946) reported oxidation of D- and L-arabinose by *P. fragi*, *P. graveolens* and *P. vendrelli*.

(*d*) *Aerobic versus anaerobic glucose utilization.* When stab cultures in Hugh & Leifson's (1953) medium were sealed with paraffin as described in Methods 165 of 169 isolates did not grow at all. The two Aeromonas isolates showed rapid growth; an acid reaction and gas bubbles developed after 18 hr. Pseudomonas isolate 55 was acid after 18 days, and isolate 36/1 showed a slight acid reaction (only in the centre of the stab) after 18 days; no growth was visible with those two isolates. Unsealed (aerobic) replicate cultures typically showed an acid reaction, developing first (within 4 days) at the surface, but completely throughout the culture after 7 days. There were a few exceptions to this; the aeromonads showed acid and gas after 24 hr.; 3 isolates grew but were not acid even after 28 days, and 7 isolates grew well but showed only a slight degree of acidity after 7 days which did not intensify on further incubation. In detail these reactions to glucose in a peptone medium did not agree completely with glucose utilization in chemically defined medium, but the usefulness of the test for the differentiation of *Aeromonas* from the obligately oxidative *Pseudomonas* as pointed out by Hugh & Leifson, was confirmed.

(*e*) *Gluconate test.* One hundred and ten isolates grew well in the Koser-type basal medium with gluconate as carbon source, and after incubation for 7 days gave a strong positive reaction after 10 min. boiling with Benedict's qualitative solution, indicative of 2-keto-D-gluconic acid production. Twenty-eight isolates grew well but gave only a weak reaction after 7 days of incubation, although 17 of these showed a good reduction after only 4 days; presumably the 2-keto-D-gluconic acid was further metabolized during the 4- to 7-day period of incubation. Of the remaining 31 isolates, 24 grew well but with no indication of reduction at 2, 4, or 7 days; 7 isolates did not grow. Haynes (1951) considered the ability to form reducing substances from gluconate as a character useful (in conjunction with others) for the identification of *Pseudomonas aeruginosa*; the present results lend no support for emphasis of this criterion. Gaby (1955) reported 2-keto-D-gluconic acid production by many strains of *Proteus* and paracolon bacteria, and Katznelson (1955) for 6 phytopathogenic *Pseudomonas* spp. and other phytopathogens.

(*f*) *Voges-Proskauer and Methyl Red tests.* All except the 7 isolates which failed to grow in the Fouad & Richards glucose salts medium were MR-positive after 7 days. This was in agreement with the known ability to produce acid from glucose by an oxidative process. All the V-P results were negative as far as could be ascertained, but on heating with 40·0% (w/v) KOH solution a bright yellow colour developed, probably due to the intensification of the colour of fluorescent pigments at the alkaline pH value, so that the results were difficult to interpret. Thus these two tests are of limited use as diagnostic criteria for pseudomonads. The V-P negative reaction of the Aeromonas isolates was not in agreement with Miles & Miles (1951) who found both these NCTC isolates to be V-P positive, but these workers used a nutrient broth medium, which may explain the discrepancy.

(*g*) *Utilization of phenol, m-cresol, naphthalene, ethanol and glycerol.* Phenol and *m*-cresol at 0·1% (w/v) were not utilizable as sole carbon sources. These tests were carried out because of reports that *Pseudomonas phenolis* n.sp. (Hamdy *et al.* 1956) could use these compounds. The general properties of this species differed from fluorescens-type organisms; e.g. *P. phenolis* grew well at 54° but not at 10° and it was very inactive biochemically. Eighty pseudomonad isolates showed a definite subculturable turbidity in the naphthalene medium, but these isolates were identical with those which grew in the control basal medium to which no carbon source had been added. Klausmeier & Strawinski (1957) obtained from soil naphthalene-oxidizing isolates which they identified as *P. aeruginosa*; Fernley & Evans (1958) reported that some Pseudomonas isolates from conifer litter oxidized naphthalene and other polycyclic hydrocarbons; utilization of naphthalene as carbon source may have occurred in my experiments.

Sixty-eight isolates grew in the ethanol medium and typically showed an acid reaction after 7 days; these were all isolates which grew in the 'minus-carbon' control medium, so the significance of this growth with ethanol seems doubtful. One randomly selected isolate (43/2) was, however, subcultured (inoculation by straight wire) more than 20 times in the ethanol medium, and dense suspensions of these organisms rapidly oxidized ethanol when tested by Warburg manometric techniques (cf. Stanier, 1947).

Glycerol was readily utilized; only 14 isolates did not grow on basal salts medium + glycerol.

(*h*) *Utilization of ammonium*-N. Only isolates 25/2, d73 and 7810 (all considered on other evidence not to be *Pseudomonas* spp.) did not grow when supplied with ammonium-N in Koser's citrate medium, although 11 isolates (mostly phytopathogens) required 3 days of incubation before developing a turbidity visible to the naked eye; the final degree of turbidity was also less than that of the other pseudomonads. However, the same isolates also showed a slower and less dense growth in a peptone + growth factor medium. The results noted in one set of control tubes (galactose + inorganic salts but no added nitrogen; potassium phosphate substituted for ammonium phosphate) were surprising: 77 isolates gave a definite acid reaction to bromocresol purple (yellow at pH 5·2) within 3–14 days. Of these isolates 88·3 % showed acidity and slight turbidity after straight-wire subculture into similar medium; 7 more isolates showed later growth but not acid production. Whether intracellular nitrogenous food reserves, atmospheric nitrogen, or nitrogenous impurities in the medium or in the air, were being used by these actively metabolizing control cultures was not ascertained. Nitrogen-fixing bacteria of the genus *Pseudomonas* appear to be common (Anderson, 1955; Proctor & Wilson, 1958). Jannasch (1958) reported that the growth of *P. fluorescens* was not limited until the nitrogen concentration was less than 0·01 mg. ammonium-N/ml. (slightly higher for nitrate- or amino-N).

(*i*) *Utilization of nitrate-N.* For all except 6 isolates turbidity developed more rapidly with ammonium-N rather than the nitrate-N in defined medium; when the final turbidities were visually compared only 16 % of the isolates showed about equal turbidities in both media. Of the isolates, 57 % showed very slight or no turbidity in the nitrate medium; this should be contrasted with the results of Seleen & Stark (1943) who found that 92·5 % of 199 strains of fluorescent pseudomonads were able to grow well on a nitrate + glucose + inorganic salts medium; they detected nitrite in 86 % of their cultures. Sometimes a slight amount of growth was obtained in the present work under anaerobic conditions; two *Pseudomonas aeruginosa* isolates and the two Aeromonas isolates showed more turbidity. Strict anaerobiosis completely prevented growth of 123 (74·6 %) isolates.

All cultures which showed any turbidity gave a positive reaction with Nessler's solution after 7 days. The results of the tests for nitrate-reduction in the chemically defined medium were the same, whether the cultures were incubated aerobically or anaerobically. Traces of nitrite were often noted; but when these are ignored (this is deemed valid because such weakly positive results were not constant on retesting) a close agreement between the results with the four test methods was evident. Only those 46 isolates which were able to reduce nitrate aerobically were able to grow anaerobically, and then only when nitrate was present. Gas production was not an absolutely constant feature, even when using the same chemically defined medium; 40 nitrite-forming isolates usually showed gas production as well, but 6 never did so. During the survey loss of nitrate-reducing ability was noted once only (isolate 42/2).

(*j*) *Utilization of nitrite-N.* Sodium nitrite at 0·05 % (w/v) was found to be non-toxic in a galactose + salts medium, although the growth of 14 % of the isolates was inhibited by 0·1 % (w/v) $NaNO_2$. Cultures in the salts + galactose + nitrite + bromocresol purple medium never showed more than a trace of turbidity, similar to that in the control medium without added nitrogen source. Thus it appeared that nitrite-N was not a suitable source of nitrogen. However when compared with growth in the control tubes it was seen that the addition of nitrite resulted in some real differences in degree of growth, namely: (i) 48 % of the isolates were able to oxidize galactose only when nitrite was supplied; (ii) 43 % of the isolates produced acid at least 48 hr. sooner in the presence of added nitrite. Nevertheless, the nitrite had not disappeared from the medium when 7-day cultures were tested with the Griess–Ilosvay reagents. Only when the complete Koser's citrate medium (containing ammonium-N) + 0·0005 % (w/v) sodium nitrite was used did 40 isolates decompose all the nitrite within 7 days. These same 40 isolates also decomposed the 0·0005 % nitrite in the semi-solid YE agar within 5 days. The microtest also showed that the same 40 isolates caused nitrite disappearance after incubation for 2 hr. Of the 46 nitrate-reducing isolates, 6 never produced gas, and these 6 isolates were unable to reduce nitrite. No isolate was found to reduce nitrite but not nitrate. The disappearance of nitrite only when some other nitrogenous source was present was observed by Seleen & Stark (1943).

(*k*) *Utilization of urea.* All isolates grew within 48 hr. on Christensen's medium and all but three developed an alkaline reaction during 2–28 days; the period differed according to the isolate. No clear differentiation of isolates was possible and it seemed likely that with such proteolytic organisms the alkaline reaction might be of double origin, from the peptone and/or from the urea.

In the low-galactose + salts + urea medium, 81 isolates did not grow. After 31 days of incubation the pH value of the remaining 88 cultures differed; 51 were alkaline, 16 were acid and 21 were approximately neutral. The 51 alkali-yielding isolates did not show a particularly rapid or intense alkaline reaction on Christensen's medium, so urea breakdown seems doubtful. Nearly all the 88 isolates apparently growing in the urea medium also developed a similar turbidity in control cultures without added nitrogenous compound; thus urea breakdown was not proven for any isolate. De Turk (1955) reported the adaptive formation of urease in washed suspensions of *Pseudomonas aeruginosa.*

Biochemical tests

(*a*) *Catalase production.* All isolates gave a definite, rapid and positive reaction indicative of the presence of catalase when 24 hr. YE agar-grown organisms were tested with hydrogen peroxide.

(*b*) *Ammonia production from peptone.* After 7 days in peptone water all cultures showed a definite turbidity and the pH values of the media were between 8·3 and 8·7 (majority 8·6; control 7·25). Every culture showed a more positive ammonia reaction than the control tube (cf. Seleen & Stark, 1943). Some of this alkalinity was probably due to ammonia production, but alkaline products of proteolysis (e.g. amines or amides) may also have been produced.

(*c*) *Haemolysis.* All isolates grew within 48 hr. on YE agar + horse-blood, but the detection of haemolysis was difficult because of the presence of pigments and partially clear zones of haemolysis. Only the two Aeromonas isolates gave zones of clear and complete haemolysis within 48 hr.; two isolates of *Pseudomonas aeruginosa* showed complete haemolysis after 3 days but *P. aeruginosa* 21 showed only a wide zone of ill-defined haemolysis. Many other isolates showed clearing underneath the growth, or a zone of partial clearing *c.* 2 mm. wide. After 5 days all plates showed complete haemolysis. Thus this test was not very useful except for differentiating Aeromonas from pseudomonads, and confirms the opinion of Liu (1957) that animal-pathogenic and non-pathogenic pseudomonads may produce haemolysis and that the character cannot be used for species identification.

(*d*) *Reaction on egg-yolk agar medium.* After incubation for 2 days a dense white precipitate surrounding the growth was apparent with 100 isolates; 23 other isolates gave a positive reaction after 3–7 days. The remaining 46 isolates grew equally well but showed no precipitate around the growth even after prolonged incubation, although clear zones (perhaps due to lipolysis or proteolysis) were sometimes apparent.

(*e*) *Gelatin hydrolysis.* All isolates except d73 grew on the nutrient gelatin medium typically on the surface only; production of pigment was only rarely seen with gelatin although on Difco Bacto gelatin good pigmentation occurred. No clear-cut demarcation between rapidly- or slowly-liquifying isolates was obvious. Regardless of whether liquifaction was stratiform or saccate, an initially rapid rate of liquifaction was not always maintained. The shape of liquifaction round the stabs was not constant for a given isolate, and this perhaps accounted for some of the apparently variable rates of liquifaction. This was further suggested in comparative experiments with nutrient gelatin stabs and streak cultures on Frazier's gelatin agar. Certain saccately-liquifying isolates apparently hydrolysed gelatin more rapidly than certain stratiform-type slower liquifiers; but all gave a 10 mm. zone of hydrolysis on Frazier's gelatin agar after 3 days.

For 67 isolates the rate of liquifaction slowed down considerably with increasing length of maintenance of stock cultures in YE broth. Eight isolates once lost the gelatin-liquifying property completely; of these the initial hydrolytic activity was not always feeble and the losses occurred at different dates. Because of the taxonomic use which has been made of this feature, the gelatin-liquifying ability of the mineral oil-preserved cultures was compared with that of the 'bench-broth' stock cultures. For 63/67 isolates the decreased rates or losses of liquifying ability when the 'bench-broths' were used for inoculation were confirmed. Once the hydrolytic power was lost by a culture it was never seen again during subsequent routine examinations.

The absence of any liquifaction after 7 days was not conclusive; frequently some hydrolysis was evident after 8–28 days. The possibility that the 1·0% peptone in the nutrient gelatin was perhaps competing with gelatin as a nutrient was considered. Twenty-eight slowly-liquifying isolates were stab-inoculated into the gelatin + yeast extract (minus peptone) medium and into ordinary nutrient gelatin simultaneously. Twenty-two isolates showed the same rate of liquifaction in both media; 5 liquified the non-peptone medium more rapidly, and one isolate showed slower liquifaction of the non-peptone medium. Further, isolates which had lost the ability to digest gelatin within 28 days on peptone-gelatin medium were not induced to do so in the absence of peptone, although surface growth was still apparent. Therefore the absence of peptone was not particularly advantageous, but the inclusion of it in nutrient gelatin was unnecessary for this collection of pseudomonads. The slowly-liquifying isolates showed signs of gelatin digestion no more rapidly on Frazier's medium. For 79 isolates the extent and rate of gelatin liquifaction remained fairly constant during 4 years. However, the importance of the gelatin-liquifying ability as a diagnostic character for *Pseudomonas* must be assessed with care. The graded series of rates of hydrolysis encountered here, from very rapid to very slow, together with the instability of the character, and the fact that different sources of gelatin may give different results, suggest that the character is not well suited for classification purposes. The writer agrees with Hormaeche & Munilla (1957) that gelatin liquifaction does not deserve the importance that has been attached to it.

(*f*) *Liquifaction of pectate gel.* Only two non-pseudomonads failed to grow in the calcium pectate stabs, but not one isolate liquified this medium (*Erwinia carotovora* effected liquefaction after 48 hr.). Pectinase and/or pectase activity has been reported for several phytopathogenic pseudomonads, e.g. (Thornberry (1938) for *Pseudomonas angulata*; Oxford (1944) for *P. marginalis* and *P. syringae*; Erikson (1945); Mills (1949); Paton (1958). My results are not in agreement because neither isolate d 263 (*P. angulata*) d 281 (*P. syringae*) nor d 289 (*P. marginalis*) showed any trace of pectate degradation here.

(*g*) *Action on milk.* In litmus milk cultures usually only a slight acid or alkaline reaction change was observed during the first 3 days. Later, pigment production and/or reduction were usual; but in 47 isolates a strong alkalinity developed, which intensified on continued incubation. When checked electrometrically, after 7 days the pH value of the blue cultures ranged between 7·1 and 7·8 (control 6·4); after 14 days the values were 7·5 to 8·3 (control 6·6). After 14 days the alkaline cultures seemed to be clearing and the determination of simultaneous proteolysis was difficult. In the absence of de-amination it was difficult to account for the alkaline reaction. The work of Ayers, Rupp & Johnson (1919) suggested an explanation, namely citrate utilization with concomitant bicarbonate formation. The milk agar plate method of testing was confirmatory: although the unmistakably proteolytic isolates effected the rapid formation of clear zones round the growth (not precipitated with acidified mercuric chloride) the intense alkali-producers gave no such indication of protein decomposition (occasional slight clearing disappeared on addition of the acidified mercuric chloride solution).

The rapid formation of a hard acid clot of milk protein was consistently characteristic only for the two Aeromonas isolates; 25 other isolates occasionally showed an acid clot, but at least 14 days of incubation were necessary. No correlation with lactose breakdown was noticed; it was concluded that because of the complex simultaneous reactions occurring in milk media, the phenomenon of hard clotting was not a very useful feature here.

Unmistakable protein digestion was visible in many neutral (or near neutral) cultures of 91 isolates after 7 days; 30 other isolates showed definite digestion during 7–28 days. The rate of digestion varied according to the isolate; sometimes it was complete after 3 days, but with other isolates it was incomplete after 28 days and it was not possible to classify the isolates except as a continuous series. Furthermore, most of the isolates showed a slowing-down of the rate of protein digestion when inoculated from stock cultures maintained on YE broth. Only for 29 isolates did the rate remain the same; in 14 isolates proteolytic activity was apparently lost altogether by the 'bench-broth' stock cultures. Further experiments with liquid cultures and the milk-agar plate method confirmed that the 'bench-broth' mother-cultures often showed much decreased rates or even complete loss of proteolytic activity toward milk, as compared with the mineral oil-preserved mother cultures.

All isolates which had lost the ability to produce proteolytic enzymes in milk were still able to grow in litmus milk with the development of marked alkalinity. They were indistinguishable from the 47 alkaligenic isolates which had always been non-proteolytic. Some isolates which initially attacked milk protein very rapidly lost this striking property, as well as isolates which originally possessed only feeble proteolytic activity.

Before, or concurrent with, the digestion of milk protein the formation of a soft rennet-type clot was usual. The medium was always nearly neutral (sometimes masked by pseudomonad pigments), and then proteolysis proceeded from the surface downwards, often resulting in banded horizontal strata in various stages of digestion. Non-proteolytic isolates never showed any rennin clotting.

The reduction of litmus or methylene blue was apparent from 48 hr. onwards in liquid milk cultures of all isolates; thus this character served no useful purpose for differentiation. Pigment production was a complicating factor and skim milk was found to be a good medium for its production. Only isolate d73 (not a pseudomonad) did not produce a change in milk.

One may conclude that milk protein digestion by *Pseudomonas* spp. is an unstable character. When freshly isolated, 71·6 % of the isolates were proteolytic and the remainder were alkaligenic (cf. 67 % proteolytic fluorescent pseudomonads in the collection described by Seleen & Stark, 1943).

(*h*) *Indole production.* Regardless of whether a peptone or chemically defined tryptophan-containing medium was used, all isolates were indole-negative except the two Aeromonas isolates. Falsely positive reactions were noted in the pyocyaninogenic cultures of *Pseudomonas aeruginosa*, due to the formation of the red salt on adding the acid reagents.

(*i*) *Production of hydrogen sulphide.* When the isolates were grown on Kligler's iron agar (Oxoid) an alkaline reaction was produced, usually after 24 hr.; the phytopathogens often required 2 days. Seven isolates caused a blackening of the medium. In the chemically defined salts + cystine medium 12 isolates (including the 7 above) produced hydrogen sulphide, but the results were not always repeatable. The microtest gave more consistent results, although incubation for 6–12 hr. was usually necessary. Again the Aeromonas isolates were distinct in that they produced hydrogen sulphide rapidly and abundantly. Apart from this, this character was of minor taxonomic value; one *Pseudomonas aeruginosa* isolate was positive, three were negative. This agrees with the conclusions of Sherwood, Johnson & Radotincky (1926); they found that only 11/22 isolates of *P. aeruginosa* blackened lead acetate agar.

The investigations concerning hydrogen sulphide production from thiosulphate were an outcome of the use of Kligler's medium which contains amino acids + thiosulphate. In the ammonium + galactose + salts + thiosulphate medium, hydrogen sulphide was produced by most cultures, but incubation for 14 days was often necessary. With the microtest incubation for *c.* 4 hr. was sufficient and the results were more consistent: 135 isolates produced hydrogen sulphide (5 weakly positive) and 34 did not. When 'oil' and 'bench-broth' stock cultures were compared it was found that 8 cultures from 'bench-broths' had lost the property.

After comparison of the results it was apparent that the character showed no obvious relationship with any other property examined here, including nitrate reduction (similar roles of nitrate and thiosulphate as hydrogen-acceptors postulated). Nor was there any relationship between hydrogen sulphide production here and in Kligler's medium or in the cystine medium. The significance of the thiosulphate-reducing reaction is not yet understood. It may be a useful diagnostic character, but the loss of the property by 8 isolates indicates some instability.

(*j*) *Hydrolysis of tributyrin.* There was extremely good agreement among the tributyrin hydrolysis results, both qualitatively and quantitatively (as measured by zone width) during four years of testing, with 5 exceptions in which the loss of tributyrinolytic activity by the 'bench-broth' stock cultures was noted, and confirmed by the retention of this property by the oil-preserved stock cultures. Incubation for 7 days was adequate and enabled detection of even the slowly hydrolysing isolates. A zone of hydrolysis of less than 2 mm. after incubation for 7 days was disregarded because of reports of limited spontaneous hydrolysis of tributyrin, and the liberation of endocellular tributyrinases on lysis (Sierra, 1957*a*, *b*). Concerning the reported toxicity of tributyrin for some micro-organisms (Collins & Hammer, 1934), although pseudomonad growth was sometimes locally sparse, a 4 mm. loopful of inoculum was sufficient to overcome the slight toxicity; only isolate d73 did not grow. Altogether 138 isolates (82·2 %) hydrolysed tributyrin and 30 did not.

(*k*) *Hydrolysis of olive oil.* Thirty-nine isolates showed no sign of blue salt formation on the olive oil medium. Thirty of these also did not hydrolyse tributyrin but of the remainder, 8 weakly hydrolysed tributyrin. Isolate d73 did not grow on the olive oil medium. Thus 130 isolates were lipolytic on both olive oil and tributyrin media. Sixteen of the olive oil-negative isolates did not grow at all; the remainder grew to an extent similar to that on control medium without olive oil. A positive reaction (heavy blue coloured growth) developed rapidly, usually after 24 hr.; but isolate 8/6 required 7 days.

(*l*) *Hydrolysis of margarine.* The similarity of the results on the olive oil and margarine medium was very marked because the same 39 isolates were non-lipolytic, and isolate 8/6 was only slowly positive on both media. The remaining 130 isolates all gave a rapid and definite positive reaction. This agrees with Goldman & Rayman (1952) who reported that *Pseudomonas* strains often differed widely in the rate and extent of hydrolysis of various fats, but rapid lipolysis was common in *Pseudomonas fluorescens*-type organisms. They also made the important observation that the susceptibility of different fats to hydrolysis depended on the particle size of the emulsion and not on the chemical composition of the fat; they standardized the globule size by means of a soy phospholipid emulsifying agent.

(*m*) *Hydrolysis of Tween* 80. The three tests carried out in 1957 showed good agreement; 120 isolates were able to hydrolyse this non-glyceride ester of oleic acid. Eighty-six isolates showed dense zones of calcium oleate crystals round the growth after 3 days; 34 other isolates were positive after 7 days. All the isolates which hydrolysed Tween 80 were also able to hydrolyse tributyrin, olive oil and margarine. However, of the 49 isolates which did not hydrolyse Tween 80 and which all grew well on the medium, 8 were able to hydrolyse tributyrin, and 31 were negative on both media. These observations agree with those of Sierra (1957*b*) and Greuell & Sierra (1957) who presented convincing evidence that *Pseudomonas aeruginosa* possesses different esterases specific for tributyrin and Tween 80, respectively, but that one enzyme, 'lipase', will hydrolyse Tween 80 or olive oil.

DISCUSSION

Many conclusions are possible from the results recorded during this survey; only the major ones can be considered here. During a period of at least 5 years the stability of each character was assessed. Even under the reasonably standardized conditions used, certain characters were not constant. These included: number of flagella/rod, colonial morphology on nutrient media or on potato slopes, pyocyanine or fluorescin production. Other characteristics were found to change when the isolates were stored in YE broth; these included the ability to digest milk protein, to liquefy gelatin, to produce hydrogen sulphide from thiosulphate, to hydrolyse tributyrin and to reduce nitrate. Frequently there was a decrease in the rate of a given reaction with increasing periods of maintenance in YE broth: this was particularly noticeable in the milk and gelatin media. Table 4 is given to show that the loss of one character was not necessarily associated with the loss of others, and that certain isolates were more unstable than others. It may be emphasized that all the results were based on a 28-day period of incubation (a disadvantage for identification purposes in diagnostic bacteriology, but essential in classification studies). Very different positive and negative results were recorded after 1, 2, 3, 4, 7, 14, 21 or 28 days of incubation.

Scrutiny of the final results showed that the following characters were common to all the isolates tested:

(1) Gram-negative, non-endospore-forming rods with an average size of $1{\cdot}5\text{–}2{\cdot}0 \times 0{\cdot}5\text{–}0{\cdot}6\,\mu$ (range $1{\cdot}0\text{–}3{\cdot}0 \times 0{\cdot}5\text{–}0{\cdot}7\,\mu$).

(2) Able to grow aerobically within 48 hr. at 25° on YE agar or YE broth initially at pH 7·2.

(3) Able to grow in YE broth + 3·0 % (w/v) NaCl.

(4) Catalase-positive.

(5) Produce ammonia when grown in peptone water medium; after 7 days the pH value of the medium is *c.* 8·6.

Table 4. *Isolates of* Pseudomonas *which showed losses of physiological characters when maintained on yeast-extract peptone medium.*

Lost character			
Milk protein digestion	Hydrolysis of tributyrin	Liquefaction of gelatin	Reduction of thiosulphate
Isolate			
.	.	.	4/2
.	.	5/5	.
5/10	.	5/10	.
.	.	8/1	8/1
.	.	.	8/9
9/4	.	.	.
.	.	.	10/3
.	.	12/3	.
12/5	12/5	.	.
15/6	.	.	.
16/1	.	.	.
18/3	18/3	.	18/3
22/2	22/2	.	.
.	22/3	.	.
22/5	.	.	22/5
35/1	.	35/1	35/1
.	.	.	36/4
.	.	40/2	.
.	40/3	40/3	.
42/1	.	.	.
.	.	.	.
47/5	.	.	.
49	.	.	.
52/1	.	.	.
52/2	.	.	.
.	.	d152	.
14	5	8	8

One isolate (42/2) lost ability to reduce nitrate

Examination of the results of the remaining tests showed that 4 isolates were distinctly different from the rest. First, isolates 7810 (NCTC; *Pseudomonas hydrophila*) and 8049 (NCTC; *P. icthyosmia*) are the only ones with the following characters: (*a*) grow anaerobically with the production of acid and gas from glucose; (*b*) no growth in the presence of the pteridine derivative O/129; (*c*) produce acid and gas aerobically from carbohydrates (glucose, galactose, L-arabinose, glycerol, mannitol, rhamnose, trehalose, sucrose, lactose); (*d*) show definite diastatic activity on starch agar; (*e*) show rapid heavy growth with acid and gas production from starch, dextrin and maltose; (*f*) produce abundant hydrogen sulphide rapidly from cystine or thiosulphate in chemically-defined media, or in Kligler's medium; (*g*) show distinct β-type haemolysis on horse-blood agar; (*h*) produce indole from peptone or tryptophan; (*i*) show rods with only one polar flagellum. These two isolates are considered to belong to a different genus, namely, *Aeromonas* Kluyver & van Niel, and will not be further discussed.

Secondly, isolate d73 (WJD; *P. mori*) was a larger and constantly peritrichously-flagellate rod. Other characters peculiar to it were: inability to grow in litmus milk, on nutrient gelatin, on potato slope, on tributyrin agar or on pectate gel; inability to use ammonium-N (e.g. no growth in Koser's citrate medium); sensitivity to the pteridine derivative O/129; markedly rough and rhizoidal colonial growth; inability to grow in YE broth of initial pH 5·5. It is difficult to place this isolate in an appropriate genus.

Thirdly, isolate 25/2 (a laboratory aerial contaminant) produced a distinct orange-yellow water-insoluble pigment when grown on YE agar, and did not utilize ammonia-N and therefore would not grow in any of the chemically defined media used here. Growth was not stimulated by the addition of 0·03% yeast extract (Difco) and it did not grow on the pectate medium. This organism is possibly a *Xanthomonas* Dowson; it was not monotrichous and had many properties in common with the rest of the collection.

Next may be considered the other properties characteristic of the remaining 165 isolates, namely:

(6) All isolates showed some rods with polar flagella, but the number of flagella/rod, amphitrichy and flagellar curvature were variable.

(7) Exocellular slime formation was typical, but the amount varied from abundant to very slight; environmental factors such as the age of culture and the nature of the medium were important controlling factors.

(8) Colonial appearance on YE agar was very variable.

(9) All were obligate aerobes; no growth occurred in glucose + peptone water under strictly anaerobic conditions.

(10) Able to grow at 12–30°; no clear-cut temperature optima.

(11) Able to grow in YE broth initially at pH 5·5.

(12) Not able to grow in YE broth initially at pH 4·5 or 4·0.

(13) Growth not inhibited by the pteridine derivative O/129.

(14) Able to use ammonia-N when supplied as the sole source of added nitrogen in an inorganic salt medium.

(15) Able to grow in Koser's citrate medium or in a similar medium with citrate replaced by sorbitol.

(16) No stimulation of growth in the simple chemically defined media used here by addition of Difco yeast extract.

(17) Not able to decompose oxalate visibly when inoculated on calcium chloride + potassium oxalate agar, although growth occurred.

(18) No significant growth (i.e. more than in the control medium without added carbon source) when starch, dextrin, cellulose, inulin, salicin or dulcitol was supplied as the sole added carbon source.

(19) Able to grow in gelatin medium (no peptone).

(20) Not able to liquefy sodium pectate gel, although able to grow on this medium.

(21) Able to grow in skim milk; litmus and methylene blue reduced.

(22) Indole negative (in peptone or tryptophan medium).

(23) Methyl red positive.

(24) Voges–Proskauer negative, but fluorescin formation masked colour reactions.

(25) Tolerated an alkaline pH value, i.e. survived more than 6 months at room temperature in YE broth at pH *c.* 8·6.

(26) Non-viable after 3 weeks at room temperature on galactose + ammonium + inorganic salts medium initially at pH 7·0.

It is concluded that, under the specified test conditions, the characteristics 1–26 above describe a fairly clearly defined group of organisms. Some of the characters have been used before in the description or definition of the genus *Pseudomonas* (see Migula, 1894; Chester, 1901; Tanner, 1918; Clara 1934; Seleen & Stark, 1943; Burkholder & Starr, 1948; Dr S. T. Cowan, personal communication; *Topley & Wilson's Principles*, 1955; *Bergey's Manual*, 1957).

The taxonomic rank accorded to any group of bacterial isolates with certain characteristics in common reflects personal opinion, and the characters cited above form a basis for an extended description and definition of the genus *Pseudomonas*. Others may show that some of the criteria are not suitable for the genus definition; e.g. oxalate-decomposing pseudomonads have been described (Bhat & Barker, 1948; Khambata & Bhat, 1953; Jayasuriya, 1955), but *Pseudomonas oxaliticus* Khambata & Bhat and the OD isolates of Jayasuriya show unusual properties for pseudomonads. Similarly, there are reports of cellulose-decomposing pseudomonads (Steel & Walker, 1957; Goto & Okabe, 1958); of diastatic strains (Lacey, 1932); of salicin-utilizers (Clara, 1934); of pectin decomposers, e.g. *P. prunicola* (Mills, 1949), some strains of *P. marginalis*, *P. syringae* and *P. medicaginis* var. *phaseolicola* (Smith, 1958). Because of such reports, these criteria should perhaps be withheld from the genus definition, but they may be useful in the separation of species. Before any of the above criteria are discarded we should be sure that the strains which make us doubt the validity of the criteria are pseudomonads and possess most of the group characters cited. The study by Sneath (1956*a*, *b*) greatly facilitates the differentiation of the closely related *Chromobacterium* genus. Another related genus, *Acetobacter*, also merits consideration when attempting to define *Pseudomonas*, particularly because of the report by Steel & Walker (1957) that two strains of *A. acetigenum* and one of *A. xylinum* gave rise to 26 celluloseless mutants which were identified as *P. geniculata*. Finally *Vibrio* must be considered when suggesting definitions for *Pseudomonas*.

It may be asked whether the group of 165 isolates defined above may justifiably be subdivided. A further 43 characters were not common to all isolates. The numbers of isolates which showed positive reactions in the 43 tests is shown in Fig. 1. Many of these characters have been used for the characterization of various species of *Pseudomonas*, e.g. *Bergey's Manual* (1957), Chester (1901), Elliott (1951), Dowson (1957). The tests were applied to the present collection with the hope that clearly defined subgroups (species?) would be revealed. Eventually this was not found to be the case. Figure 1, which summarizes the results, does not reveal the fact that there was no correlation between any two of the 43 characters mentioned; the negative isolates in one

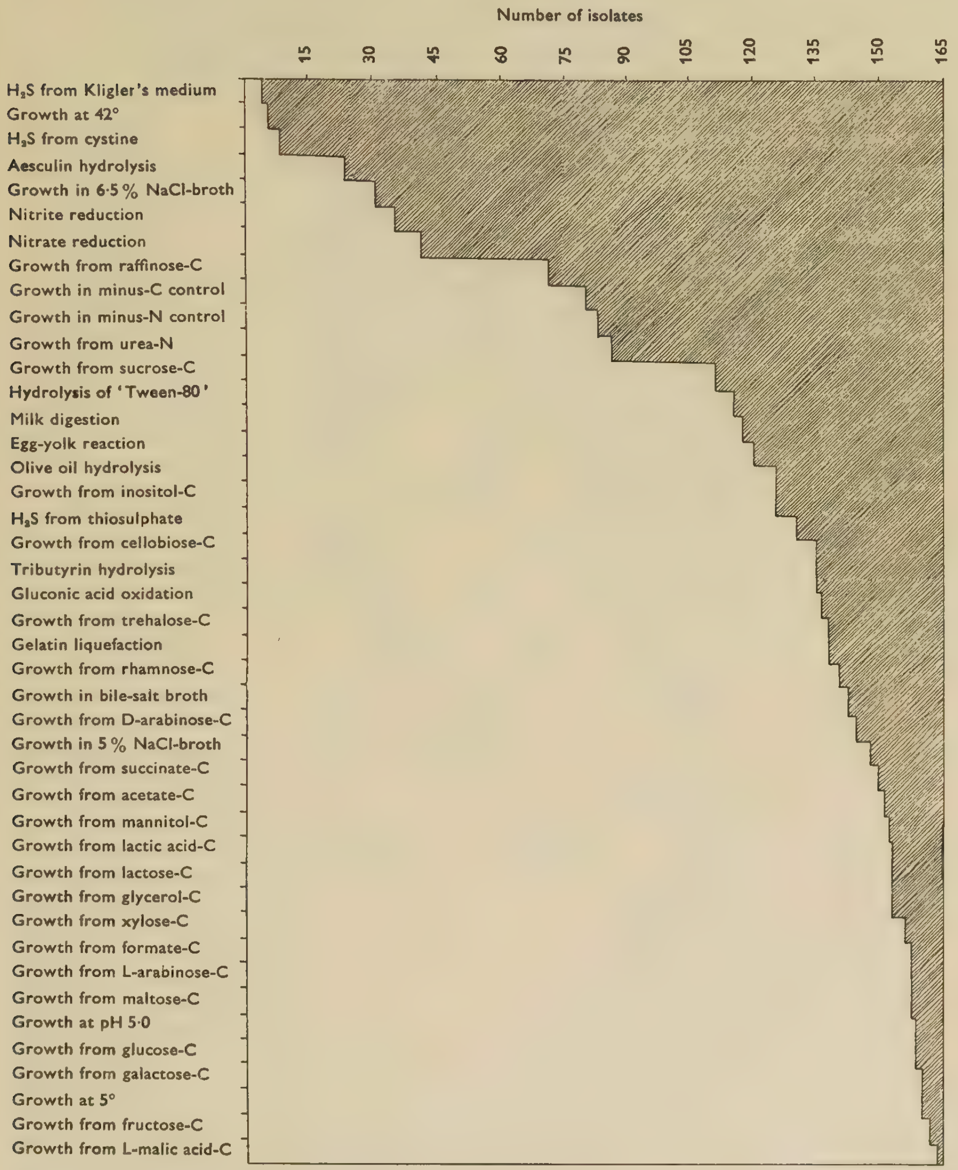

Fig. 1. Histogram showing the number of positive and negative test results when 165 isolates of *Pseudomonas* were tested for 43 characters. These 43 characters were chosen because they were not common to the whole group of 165 isolates; this group was definable on the basis of 26 other characters. See text for details of all the test characters used. Hatched areas indicate negative results; clear areas indicate positive test results.

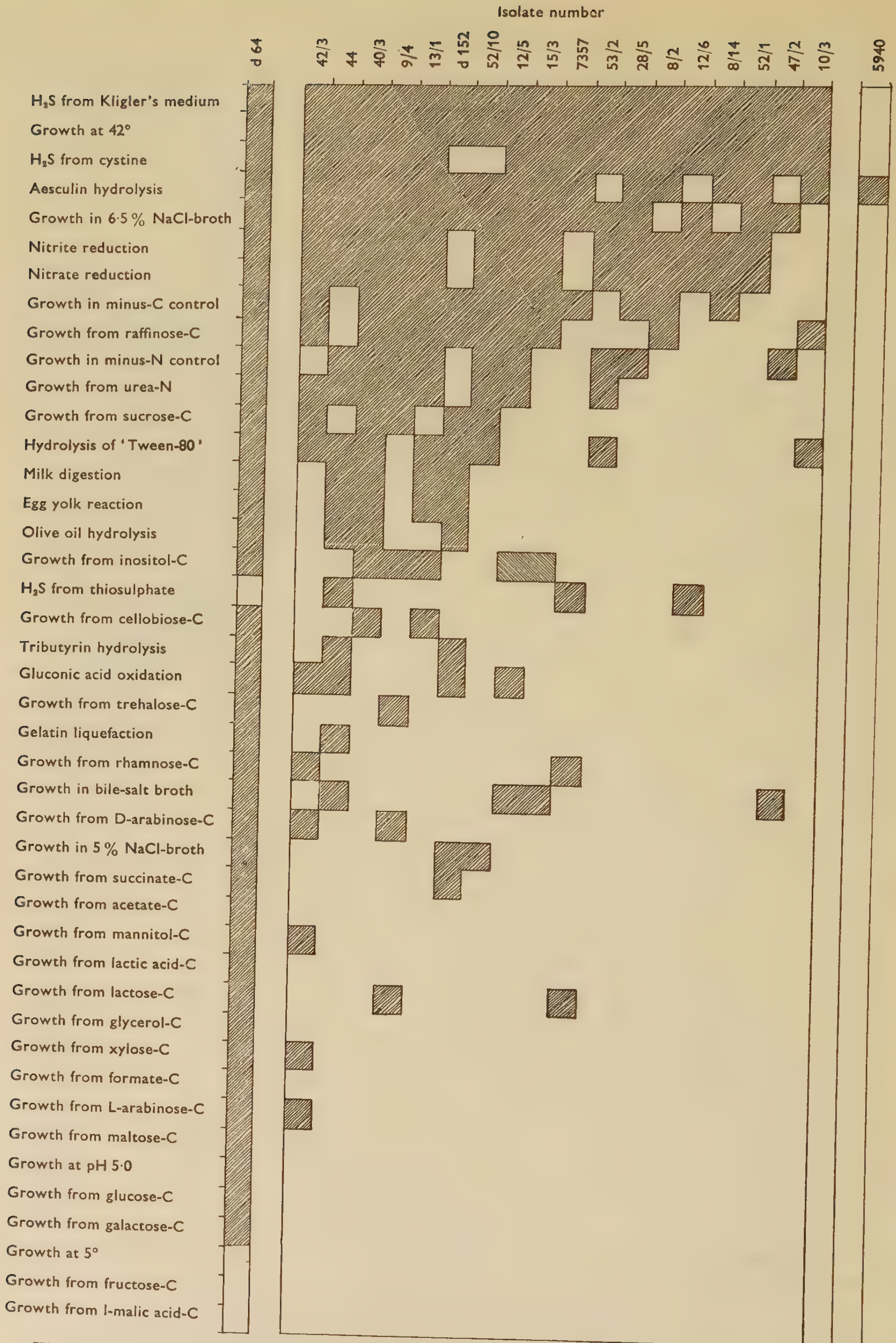

Fig. 2. Illustration of the range of variation shown by a random selection of *c.* 10·0 % of the isolates of the pseudomonad collection concerning 43 differential test characters. Isolates no. 5940 (*P. fluorescens*, NCTC 5940) and d64 (*P. savastanoi* from Dr W. J. Dowson) are also included for comparison: no. 5940 was the most reactive isolate of the whole collection and d64 the most non-reactive. For details of test characters, see text. Hatched areas indicate negative results; clear areas indicate positive test results.

column were not identical with those in any other. One isolate gave positive reactions in 42 of the 43 characters, and one was negative for 39 characters: the remaining isolates could be arranged between these extremes in an order of a gradually increasing number of negative characters. Among 58 isolates there were 21 small groups of identical isolates, but the largest number in any group was 5; the remaining 107 isolates each had a different combination of the 43 characters. A similar situation emerged in the taxonomic study of aquatic fluorescent pseudomonads by Tanner (1918); he suggested the use of more

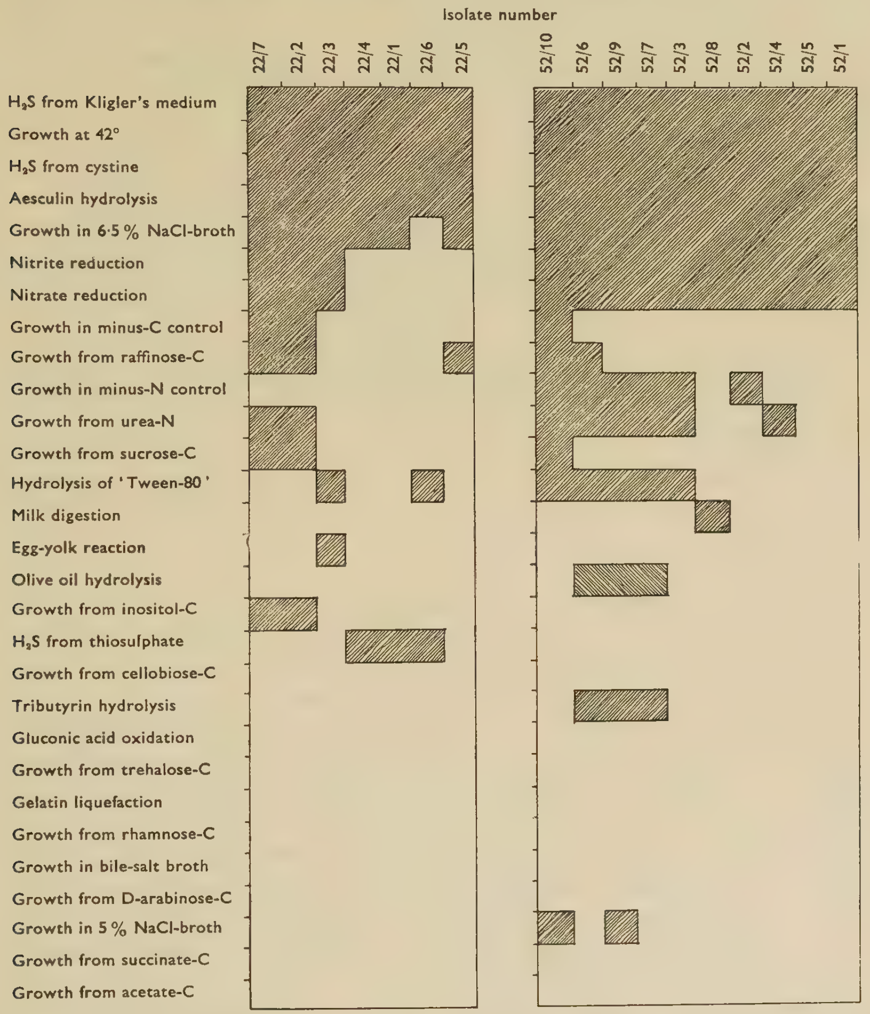

Fig. 3. To show the range of variation concerning 29 test characters, exhibited by the 10 pseudomonad isolates of the no. 52 series; each isolate was originally obtained from a single fluorescent pigment-producing colony, and all 10 isolates were picked from one plate of nutrient medium which had been streaked with one 4 mm. loopful of river water. Series no. 22 similarly shows the properties of 7 isolates all originally derived from 1 ml. raw milk. See text for details of the test characters. Hatched areas indicate negative results; clear areas indicate positive test results.

refined experimental techniques as the remedy, but perhaps these were already too refined. His use of the 'Group Number' system as a device to express equal emphasis on all the diagnostic characters used is noteworthy. The same approach has been taken here (see Sneath, 1957*a*, *b*, for the argument concerning the validity of this Adansonian approach).

Full tables of the results obtained in this work, involving 165 isolates and 43 characters cannot be given here; the complete data are available in this Department. All the data have been arranged and sorted in many ways as objectively as possible, giving equal weight to every character, to see whether there were any larger groups with at least two correlated characteristics in common. None could be found. To convey the great range of variation among the isolates Fig. 2 is given as a sample of the picture shown by *c.* 10·0 % of the isolates, randomly selected.

Table 5. *Showing the eight variable properties exhibited by the five isolates of* Pseudomonas aeruginosa *tested*

The characters of two *P. fluorescens*-type isolates are given for comparison. Thirty-seven other characters we positive for all seven isolates (see text). + indicates a positive result; − indicates a negative result.

Isolate	Pyocyanine production	Fluorescin production	Growth at 42°	H_2S from Kligler's medium	H_2S from cystine	Aesculin hydrolysis	H_2S from thiosulphate	Growt at 5°
5940	−	+	+	+	+	−	+	+
20	+	+	+	+	+	−	−	−
21	−	+	+	+	+	−	−	−
24/2	+	+	+	+	+	−	−	−
24/1	+	+	+	+	−	−	−	−
36/4	−	+	−	−	−	−	+	+
8/11	−	+	−	−	−	−	−	+

Another illustration of the variation exhibited by the isolates with regard to these 43 properties is given in Fig. 3. Here the 10 isolates of the no. 52 series (which were all orginally isolated as separate fluorescent pigment-producing colonies on one plate of YE agar which had been streaked with one loopful of River Kennet water) are shown. Series no. 22, with seven isolates similarly derived from 1 ml. of a raw milk sample, is also shown. It is therefore concluded that the 43 properties are not useful for the definition of subgroups because of their complete lack of correlation with each other. Furthermore, some of these properties are unstable under certain conditions and when adequately tested it seems probable that many more would be found to be unstable. Further conclusions which emerged from an examination of the complete data are as follows.

(1) There was a group of 5 isolates able to grow at 42° and to produce hydrogen sulphide on Kligler's medium. These were positive in all the other 41 characters, with few exceptions (see Table 5). In many respects this group conforms with the revised characterization of *Pseudomonas aeruginosa* (Haynes, 1951; Gaby, 1955). Too few of these isolates of this accepted species have been examined here to warrant further comment, but the properties of isolates 8/11

and 36/4 (in Table 5) indicate the close overall similarity of some *P. fluorescens*-type isolates when a total of 45 characters is considered. Liu (1952) claimed that *P. aeruginosa* showed a definite biochemical pattern of carbohydrate utilization; this pattern was given by many *P. fluorescens*-type isolates of the present collection. Brown (1957) concluded that there were no obvious distinctions between *P. aeruginosa* and psychrophilic *P. fluorescens*-type organisms, but he found that the latter were able to multiply much sooner over nearly the whole temperature range in which growth of both species occurred, suggesting the greater adaptability of the psychrophil in a new and more difficult environment. Furthermore, the psychrophil showed a much more vigorous glucose-oxidizing ability over a wider range of temperature, and a greater acid tolerance. This is in keeping with my view that *P. aeruginosa* is more likely to be derived from a less specialized 'wild type' such as *P. fluorescens*, rather than the reverse.

(2) Isolate d289 (WJD, *Pseudomonas marginalis*) was identical with isolates 47/1 and 47/4 (from a vase of tap water containing a *Colchicum* flower), except that the latter two were unable to grow in bile salt broth. Isolate d52 (WJD; *P. medicaginis* var. *phaseolicola*) was identical with isolates 52/1 and 52/5 (from River Kennet water). Isolate d263 (WJD; *P. angulata*) was identical with isolate 7/3 (from a pond) and isolates 8/2 and 8/15 (from human plasma); all these had the same 8 negative characters. Isolate d244 (WJD; *P. barkeri*) was identical with isolate 25/1 (from the air of a veterinary laboratory) and these two had the same 12 negative characters. This suggests either that phytopathogens may be found in a wide variety of natural habitats, or that phytopathogenicity is not associated with any particular metabolic or cultural pattern so far investigated. Phytopathogenicity tests on all these isolates might profitably be attempted; Clara (1934) found that *P. fluorescens* caused mild disease symptoms on Kieffer pears; Stapp (1955) reported that a strain of *P. aeruginosa* was pathogenic to potato tubers; *P. polycolor* (Elrod & Braun, 1942) is a well-known facultative plant and animal pathogen. The close similarity between phytopathogenic and saprophytic pseudomonads, such as described by St John Brooks, Nain & Rhodes (1925), and by Friedman (1953) who used physiological and serological criteria, is thus accidentally confirmed.

(3) Of the remaining named phytopathogens 11 had between 23 and 39 negative characters (of the 43 under consideration). They occupied positions almost exclusively at the lower end of a table arranged to show isolates with an increasing number of negative characters, suggesting a relationship between phytopathogenicity and a decreased ability to withstand inimical agents such as sodium chloride, bile salts or acids. Also, fewer carbon sources were utilized, and 7 of these isolates did not grow with glucose as carbon source; nearly all the biochemical reactions were negative too, and the rate of growth on complex nutrient medium was also slower. This perhaps again indicates decreased physiological adaptability, compared with the 'wild-type' *P. fluorescens*, as being concomitant with effective pathogenicity.

(4) When the named isolates of this collection (those obtained from diseased plant material and the *P. aeruginosa* group) are omitted, the following further

characterization applied to the remaining group of 134 isolates from saprophytic soil- and water-type environments (initially they produced fluorescin):

(*a*) Did not produce hydrogen sulphide from Kligler's medium. (*b*) Would not grow at 42° or 37°. (*c*) Would grow well at 5°. (*d*) Would grow in YE broth initially at pH 5·0. (*e*) Would utilize glucose, galactose, fructose, maltose, l-malic acid and lactic acid when these were supplied as added carbon sources in a Koser-type defined basal medium.

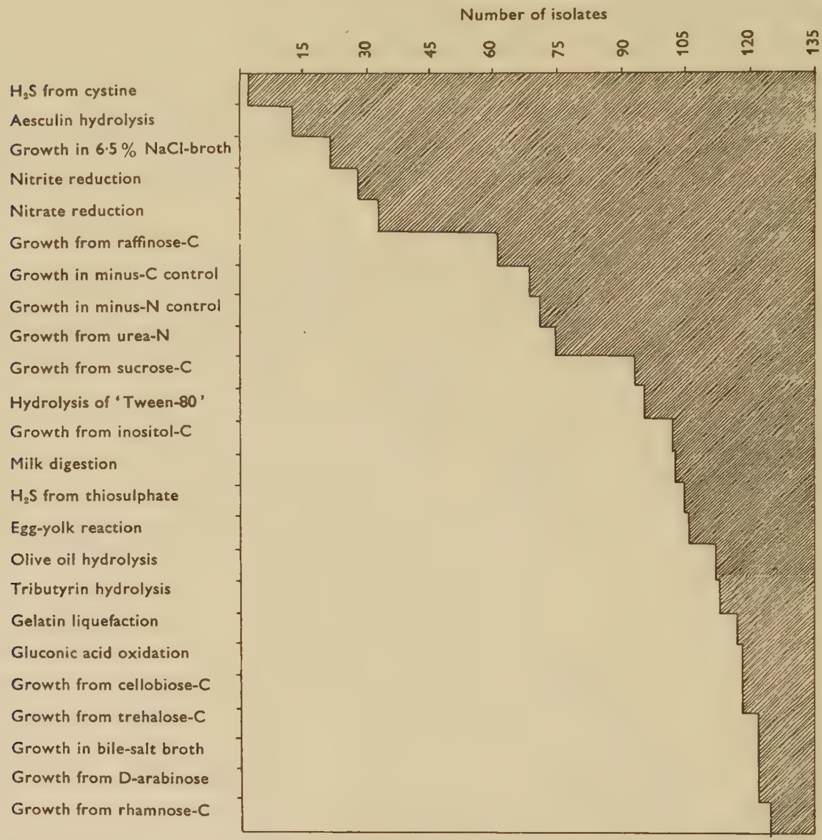

Fig. 4. Histogram showing the number of positive and negative test results obtained when 134 *Pseudomonas* isolates, all soil and water types initially producing fluorescin, were examined by means of 24 differential test characters. See text for full details of tests. Hatched areas indicate negative results; clear areas indicate positive test results.

In a further 9 tests only the following isolates (bracketed) showed a negative reaction: glycerol (43/1), succinate (10/1), L-arabinose (42/2, 42/3), formate (10/1, 27/3), acetate (10/1, 27/3), lactose (9/4, 13/4), xylose (9/2, 42/3), mannitol (42/2, 42/3, 43/4), ability to grow in YE broth + 5·0 % NaCl (5/5, 8/6, 42/2, 52/9, 52/10). The isolate numbers are given to show that these non-conformities were not correlated; they were shown by only 13 different isolates.

The numbers of the 134 fluorescent isolates which showed positive reactions in the remaining 24 tests are given in Fig. 4. No correlation of any of the characters was evident and further subdivision of this group is thus not considered to be practicable or justified, because, for example, the same 122 isolates were not positive in the D-arabinose, trehalose and bile salt broth tests.

Thus an accurate description of commonly occurring pseudomonads can be made. From the full data mathematically inclined taxonomists might well derive further conclusions, but I put forward, from the work described here, the following description of the genus *Pseudomonas*, and by using further characters give a revised definition of *Pseudomonas fluorescens* Migula (see also the *Int. Bull. Bact. Nomen. & Taxon.* 1951, 1952). This description is:

Genus *Pseudomonas* Migula, 1894

Gram-negative non-endospore-forming rods with size range $1{\cdot}0\text{–}3{\cdot}0 \times 0{\cdot}5\text{–}0{\cdot}7\,\mu$. Able to grow aerobically within 48 hr. at 25° on yeast extract + peptone media (initial pH 7·2) with a sodium chloride concentration up to 3·0 %. Catalase-positive. Produce some free ammonia when grown in peptone water medium, and after 7 days at 25° the medium is alkaline (*c.* pH 8·6). Polar flagella (1–10) and exocellular slime present. Colonial appearance on complex yeast extract + peptone or nutrient media very variable. Obligate aerobes; no growth in glucose + peptone water under strictly anaerobic conditions. Able to grow at 12–30°; no clear-cut temperature optima. Able to grow in complex nutrient media (initially pH 5·5) but not able to grow when initial pH value is less than 4·5. Resistant to the pteridine derivative O/129. Able to use ammonia-N when it is supplied as the sole source of added nitrogen in a suitable inorganic salts basal medium. Able to grow in Koser's citrate medium or in a similar basal medium with the citrate replaced by sorbitol. No factor in Difco yeast extract stimulates growth in the simple chemically defined media used here. No significant growth (i.e. more than in control medium without added carbon source) when dulcitol is supplied as the sole added carbon source. Able to grow on gelatin medium (no peptone) and on potato slopes. Able to grow in skim milk with reduction of litmus or methylene blue. Indole negative. Methyl red positive. Voges–Proskauer negative (but fluorescin may mask colour reaction). Tolerate an alkaline pH value, i.e. survive more than 6 months at room temperature in YE broth with a pH value 8·5 at 14 days. Non-viable after 3 weeks at room temperature on ammonium + inorganic salts + galactose medium (initially pH 7·0).

Species *Pseudomonas fluorescens* Migula, 1894

This species possesses all the properties of the genus as described above, together with the following characters: will not grow at 42° or 37°; will grow well at 5°. Will grow in complex nutrient media of pH 5·0. Grows, but does not produce hydrogen sulphide, on Kligler's medium. Will utilize glucose, galactose, fructose, maltose, l-malic acid or lactic acid when these are supplied as the sole added carbon source in a Koser-type inorganic salt medium.

The following properties may be positive or negative (the number in brackets refers to the % of isolates in this collection which showed a positive reaction): utilization of glycerol (99), succinate (99), L-arabinose (98), formate (98), acetate (98), lactose (98), xylose (98), mannitol (98), rhamnose (93), D-arabinose (91), trehalose (91), cellobiose (88), inositol (75), sucrose (70), raffinose (46), when these substrates are supplied as the sole added carbon source in ammonium + inorganic salts medium; growth in peptone yeast-extract + 5·0 % (w/v) NaCl medium (96); ability to grow in MacConkey's bile salt broth (91); production of 2-keto-D-gluconate acid from D-gluconate (88); liquefaction of gelatin (87); production of an egg-yolk reaction (79); production of hydrogen sulphide from thiosulphate (78); digestion of milk protein (76); hydrolysis of Tween 80 (72); production of definite turbidity in an inorganic salts medium without an added nitrogen (53), or added carbon source (52); reduction of nitrate (25); reduction of nitrite (21); growth in yeast-extract + peptone + 6·5 % (w/v) NaCl medium (16); hydrolysis of aesculin (10); production of hydrogen sulphide from cystine (1).

Note. The production of fluorescent pigment (or indeed any pigment) is not used in the above definitions because it is generally admitted that the property is unstable and dependent on the nature of the medium for its manifestation (see Seleen & Stark, 1943, for review). Moreover, greenish yellow fluorescent pigment production by non-pseudomonads has been recorded, e.g. by *Bacillus subtilis* (Shank, Chmura & Silliker, 1958).

The description of *Pseudomonas fluorescens* in the latest (7th) edition of *Bergey's Manual* (1957) differs from the above in many details. Also, if the definition of *P. fluorescens* suggested here be acceptable, many of the Pseudomonas species listed in the *Bergey's Manual* (1957) appear to be unjustified. In the absence of adequate data concerning some organisms, judgement is postponed, but the writer feels that the following species descriptions agree well with the *P. fluorescens* described above, namely, *P. schuylkilliensis, geniculata, septica, incognita, mildenbergii, convexa, eisenbergii, cohaerens, denitrificans.* The following list of species seems to possess only minor differences: *P. syncyanea, tetraolens, rugosa, putrefaciens, striata, ovalis, mephitica, mira, putida.* Gaby (1955) thought that *P. mildenbergii* and *P. ovalis*, and *P. fluorescens* and *P. putrefaciens* were indistinguishable pairs. Clara (1934) found that *P. putida* and *P. fluorescens* differed only in minor characters. The above suggested description for *P. fluorescens* agrees much more closely with the *P. fluorescens* as described by Clara than with that of *Bergey's Manual.* Seleen & Stark (1943) also showed a close similarity between *P. denitrificans, putida, ovalis* and *convexa.* Ideally, comparison of named cultures (when available) and freshly isolated cultures of all these species, using standardized techniques, is necessary so that some of the 'taxonomic problems' outlined by Gaby (1955) concerning the identification of *Pseudomonas* species may be clarified. The literature concerning the use of serology for *Pseudomonas* differentiation was reviewed by Munoz, Scherago & Weaver (1949); their own findings were encouraging in that their three strains of *P. fluorescens* were serologically similar (contrast serological heterogeneity of *P. fluorescens* reported by Friedman, 1953), but they concluded that *P. fluorescens, mildenbergii, ovalis* and *putida* showed no serological relationships; they were quite distinct on the basis of flagellar antigens. Thus is posed a fundamental problem of bacterial classification, i.e. the status of a bacterial species, and whether this taxonomic rank should be assigned to organisms which may be distinguished only serologically (Kauffman, 1959).

A neotype isolate of *Pseudomonas fluorescens* has been selected (no. 2/85, see Fig. 2 here) and deposited in the National Collection of Type Cultures, Colindale, London, England (no. 10,038) and in the American Type Culture Collection (no. 13,525).

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Protozoa as Pharmacological Tools: The Antihistamines

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SUMMARY: Two protozoa, *Ochromonas malhamensis* and *Tetrahymena pyriformis*, were used to study the mode of action and side effects of three antihistamines: diphenhydramine, tripelennamine and pheniramine. The mode of action was studied by inhibition analysis and the side effects by motility tests. The use of these methods as preliminary screening procedures for new chemotherapeutic agents is discussed.

Goals in experimental pharmacology include not only the improvement of existing chemotherapeutic agents, but also the elucidation of the metabolic loci of action of these agents. It is desirable to construct model microbiological systems which would parallel and isolate the key animal reactions without the extraneous variables contributed by the use of higher animals. We report here on a series of protozoon assay methods designed to show with antihistamines drug potency, mode of action, toxicity to the host and side actions.

METHODS

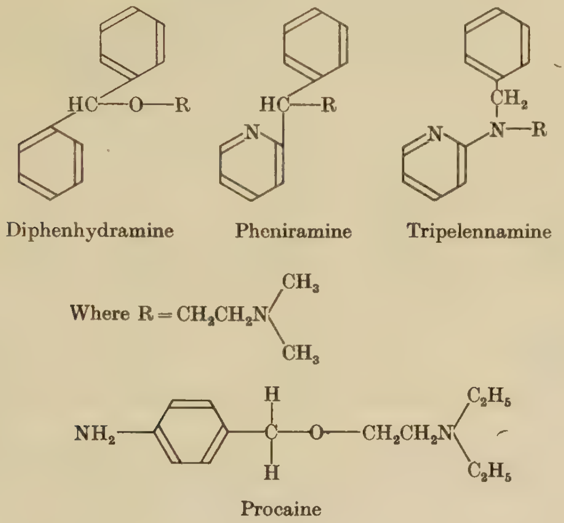
Tetrahymena pyriformis, a ciliated protozoon which may be grown in defined media and which has an exogenous requirement for histidine, was used for growth and motility studies. The maintenance, inoculation and experimental media were those described by Stokstad, Seaman, Davis & Hutner (1956).

Ochromonas malhamensis, which also has an exogenous requirement for histidine, was only used for growth experiments. Experiments were done either at pH 5·0 in the medium given in Table 1 of Hutner *et al.* (1957) or at pH 7·0 in the medium given in Table XI of Nathan & Funk (1959).

In growth experiments, both *Tetrahymena pyriformis* and *Ochromonas malhamensis* were allowed to grow until the end of the logarithmic growth phase. Growth was then measured as optical density in a Welch Densichron equipped with red probe. The 'potency' of the drugs was evaluated as degree of growth inhibition. The sites of the biochemical lesions were located by the ability of certain compounds to annul this inhibition.

Diphenhydramine (Benadryl), tripelennamine (Pyribenzamine), and pheniramine (Trimeton), all obtained from commercial sources, were used. These compounds were selected as representative members of the three major classes of antihistamines based on whether carbon, nitrogen, or oxygen serve as the link between the aromatic and aliphatic portions of the antihistamine molecule (Fig. 1). The structural similarity of diphenhydramine to procaine (Fig. 1), which possibly accounts for the former's local anaesthetic properties, suggested procaine as a control for the motility studies.

Two-day log-phase cultures of *Tetrahymena pyriformis*, which had been grown in the defined medium, were used for motility experiments. The organisms were separated from the medium by centrifugation and diluted with 0·85% (w/v) NaCl solution (saline) to yield a suspension with an optical density of about 1·0. Each well of a depression slide contained 0·15 ml. of this suspension + the experimental compounds (in solution adjusted to pH 7·0) + saline to bring the volume in each well to 1·0 ml. The effects of the test compounds on the motility of the organisms in the well were observed by direct microscopic examination. Each motility experiment was continued until the organisms in the well containing the procaine control ceased all motion; see, for example, the duration of the two experiments noted in Table 3.



Diphenhydramine Pheniramine Tripelennamine

Where $R = CH_2CH_2N(CH_3)_2$

Procaine

Fig. 1

RESULTS AND DISCUSSION

The multiplicity of the sites of action of antihistamines has been noted many times. Respirometric and growth experiments on toxicity and the annulment of the toxicity of antihistamines have produced a variety of results, e.g. Landis & Krops (1951) reported annulment of the fungistatic action of antihistamines on *Trichophyton* spp. by histamine but not histidine, but Reiss & Caroline (1955) found that neither histidine nor histamine would annul pheniramine toxicity in *Blastomyces* sp. Di Ritis & Zanussi (1949) showed annulment of antihistamine toxicity for bacteria by thiamine + nicotinic acid. Carlisle & Crescitelli (1950) reported antihistamine inhibition of rat brain cortex respiration when glutamate, but not glucose, served as carbon source. The local anaesthetic effects of antihistamines were reported by Steffen, Zimmerman & Mihan (1956) and suggested another activity of antihistamines which might be investigated with protozoa.

Our first experiments were done with *Ochromonas malhamensis*, which has been shown to parallel animal specificity for vitamin B_{12}, and, more pertinent to these studies, in response to at least one form of stress, namely heat (Hutner *et al.* 1957). As others have shown that antihistamines are inactive below pH 6·8, possibly from failure of the drugs to penetrate cell membranes because of their ionization state or poor solubility (Jahn & Danforth, 1952), it was not surprising that when *O. malhamensis* was grown at pH 5·0 relatively high concentrations of antihistamines (0·3–1·0 mg./ml.) were needed to inhibit growth. 'Loading' of the system with antihistamines at pH 5·0 obscured the fine biochemical sites of drug action; the inhibition was annulled non-specifically by carbohydrates, energy-yielding amino acids and metals. This could be used as an indicator of host toxicity induced by drug overdose. The comparison of drug toxicity levels for diphenyramine and tripelennamine at pH 5·0 (0·3–1·0 mg./ml.) and at pH 7·0 (0·01–0·03 mg./ml.), where the specific inhibitory sites of the drugs are evident (Table 1), gives a rough estimation of the therapeutic index to expect for these drugs. This gross comparison agrees well with the clinical therapeutic indices. The results obtained in the motility experiments used to demonstrate side actions of the drugs also paralleled the degree of clinical side action (drowsiness) of each of these drugs.

Table 1. *Inhibition of growth* of* Ochromonas malhamensis *by antihistamines*

	Drug additions (mg./ml.)						
		Benadryl		Pyribenzamine		Trimeton	
	None	0·003	0·01	0·003	0·03	0·1	0·3
I. Additions in μg./ml.							
None	1·96	1·73	0·22	1·94	0·13	1·15	0·28
DL-Ornithine 0·3	1·97	—	—	1·94	0·24	1·06	0·64
DL-Ornithine 1·0	1·89	1·52	0·33	—	—	—	—
L-Proline 0·3	1·60	0·96	0·49	—	—	1·22	0·84
L-Histidine 0·3	2·00	1·68	0·90	1·98	0·42	1·41	1·27
L-Histidine 1·0	1·71	1·50	0·94	1·67	1·49	1·39	1·13
II. None	1·64	—	0·06	—	0	1·35	—
DL-Ornithine 0·1	1·79	—	0·30	—	0·33	1·78	—
L-Proline 0·05	—	—	—	—	—	—	—

* Growth expressed optical density units in this and subsequent tables. pH 7·0.

The similarity of results obtained with *Ochromonas malhamensis* grown at pH 7·0 (Table 1) and in growth experiments with *Tetrahymena pyriformis* (Table 2) indicates that either microbial system may be used to seek evidence for metabolic action sites. In both cases histidine was most active in annulling drug inhibition. Chromatographically pure histamine did not annul drug inhibition. The activity of the impure histamine preparations might be explained by the presence of a histidine contaminant. Ornithine and proline, used singly, were moderately effective in annulling toxicity for both organisms. Together these amino acids were as effective as histidine for *T. pyriformis* (Table 2) but not for *O. malhamensis*. For *T. pyriformis* and for *O. malhamensis* there were some annulment effects by calcium, iron, magnesium and thiamine + nicotinic acid, but these were all less effective than the above amino acids. Hydroxyproline, urocanic acid, glutamic acid, tryptophan, alanine and pyridoxamine, all of which are metabolically related to histidine, ornithine and proline, were inactive.

The principal clinical side action of antihistamines, drowsiness, was also studied microbiologically. The highly motile ciliates have an elementary neuromotor system consisting of a co-ordinating centre from which fibres radiate to different ciliary structures at whose bases are found basal granules to which the 'nerve fibres' are attached (Kudo, 1950). *Tetrahymena pyriformis* was selected to demonstrate the protozoan version of the soporific and local anaesthetic actions of antihistamines (Table 3).

The clinical observation that, of the three drugs used in our experiments, pheniramine exhibits fewer side effects, was paralleled in our protozoan motility experiments (Table 3). Here the intensity of side effects may be ranged: diphenhydramine > tripelennamine > pheniramine.

Table 2. *Inhibition of growth of* Tetrahymena pyriformis *by tripelennamine*

	Tripelennamine* (mg./ml.)				
	None	0·005	0·01	0·0125	0·015
I. Additions in μg./ml.					
None	1·33	1·35	0·04	0	0
DL-Ornithine 1·0	1·58	1·62	1·23	0	0
L-Proline 0·05	1·41	1·39	1·47	0·21	0
L-Histidine 0·3	1·18	1·20	1·33	0·55	0
L-Histidine 1·0	1·25	1·25	1·01	1·00	1·14
L-Proline 0·05 + DL-Ornithine 1·0	1·58	1·53	1·66	1·45	1·24

* Results with diphenhydramine and pheniramine essentially the same.

Table 3. *Effect of antihistamines and of procaine on the motility of* Tetrahymena pyriformis

Experiment 1

	Elapsed time (min.)						
	0	3	9	11	17	22	36
(1) Additions in mg./ml.							
No additions	4+	4+	4+	4+	4+	4+	4+
Glucose 2·0	4+	4+	4+	4+	4+	4+	4+
L-Histidine 2·0	4+	4+	4+	4+	4+	4+	4+
(2) Procaine 2·5 plus							
No further additions	4+	2+	1+	±	±	0	0
Glucose 2·0	4+	4+	2+	1+	±	0	0
L-Histidine 2·0	4+	4+	4+	2+	2+	±	±
(3) Diphenhydramine 0·25 plus							
No further additions	4+	4+	2+	±	±	±	0
Glucose 2·0	4+	4+	3+	1+	±	±	0
L-Histidine 2·0	4+	4+	4+	2+	2+	2+	2+
(4) Tripelennamine 0·25 plus							
No further additions	4+	3+	2+	±	±	±	0
Glucose 2·0	4+	3+	2+	±	±	±	0
L-Histidine 2·0	4+	4+	3+	2+	2+	2+	2+

Table 3 (*cont.*)

Experiment 2

	Elapsed time (min.)		
	3	53	106
(1) Additions (mg./ml.)			
No additions	4+	4+	4+
Glucose 10·0	4+	4+	3+
L-Histidine 5·0	4+	4+	3+
Glucose 10·0 + L-Histidine 5·0	4+	4+	4+

	Procaine (mg./ml.)					
	0·5	5·0	0·5	5·0	0·5	5·0
(2) No additions	4+	3+	3+	1+	1+	0
Glucose 10·0	4+	2+	3+	±	2+	0
L-Histidine 5·0	4+	4+	3+	3+	2+	2+
Glucose 10·0 + L-Histidine 5·0	4+	4+	3+	2+	3+	2+

	Diphenhydramine (mg./ml.)					
	0·1	0·3	0·1	0·3	0·1	0·3
(3) No additions	4+	4+	3+	1+	2+	±
Glucose 10·0	4+	3+	2+	2+	2+	1+
L-Histidine 5·0	4+	4+	3+	3+	3+	2+
Glucose 10·0 + L-Histidine 5·0	4+	4+	3+	2+	3+	2+

	Tripelennamine (mg./ml.)					
	0·1	0·3	0·1	0·3	0·1	0·3
(4) No additions	4+	4+	3+	2+	1+	1+
Glucose 10·0	4+	4+	3+	3+	2+	1+
L-Histidine 5·0	4+	4+	3+	4+	2+	3+
Glucose 10·0 + L-Histidine 5·0	4+	4+	3+	3+	3+	3+

	Pheniramine (mg./ml.)					
	0·1	0·3	0·1	0·3	0·1	0·3
(5) No additions	4+	4+	3+	3+	2+	2+
Glucose 10·0	4+	4+	3+	2+	3+	2+
L-Histidine 5·0	4+	4+	4+	4+	3+	3+
Glucose 10·0 + L-Histidine 5·0	4+	4+	3+	4+	3+	4+

4+ = No organisms rounded up, most are actively motile.
3+ = >90 % of organisms motile, few are rounded up.
2+ = 50 % of organisms motile, 50 % are rounded up.
1+ = 75 % of organisms rounded up, sluggish motility in the rest.
± = all organisms rounded up.
0 = most organisms exploded, the remainder rounded up.

Histidine was most effective in maintaining motility in the presence of the antihistamines (Table 3). Glucose, a non-specific substrate control, had slight activity alone and did not significantly enhance the effectiveness of histidine. The histidine-contaminated histamine partially maintained motility in the presence of pheniramine and tripelennamine but was inactive in annulling the effects of procaine or diphenhydramine, probably because there was only enough histidine present (as contaminant) to counteract the compounds which had weak anti-motility action. In the growth and the motility experiments, diphenhydramine and tripelennamine were *c.* 5 times as active as procaine in the motility experiments. Pheniramine was considerably less inhibitory than diphenhydramine or tripelennamine in growth and in motility experiments. The efficacy of histidine in annulling antihistamine inhibition suggests that it is biochemically close to the 'active' material whose metabolism is blocked by the antihistamine. That this same 'active' substance is involved in nerve conduction is implied by the work of Crescitelli & Geissman (1951) which demonstrated an antihistamine produced conduction block in frog sciatic nerve and by the results of our motility studies with *Tetrahymena pyriformis*. It is not yet possible to separate the role that histidine may play in nerve conduction, as a metabolic intermediate, and as a metal chelator; indeed there may be some overlap. As known intermediates in the pathway between histidine, ornithine, and proline were inactive in annulling antihistamine growth-inhibition, another, as yet undescribed metabolic connexion among these three may explain their activity.

The histamine which is bound in mammalian tissues is formed from L-histidine but Schayer (1956) noted that it has never been possible to demonstrate binding of exogenous histamine in intact animals. The lack of activity of histamine in our experiments may be a reflection of the inability of the protozoon, like the mammal, to metabolize exogenous histamine.

By combining the results of our growth and motility experiments, it should be possible to suggest that an antihistamine in which the side chain is attached to the aromatic nucleus via a carbon link rather than an ether link would be low in soporific action and that small alterations in this molecule might produce a more effective antihistamine with minimal side effects. In addition, the anti-motility effects observed were quantitatively similar both to the soporific side effects and the local anaesthetic properties of the antihistamines in humans. An approximate margin of safety (therapeutic index) and even a specific antidote might also be suggested. While the efficacy of these antihistamines in annulling growth inhibition was not quantitatively analogous to the efficacy in human beings, the final test for all pharmacological agents must be clinical trial, and, in this respect, the system shares the shortcomings of other preclinical methods.

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Fimbriae and Adhesive Properties in Klebsiella Strains

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SUMMARY: Fimbriae were found in 125 of 154 non-motile Klebsiella strains examined by electron-microscope in serial aerobic broth cultures. Fimbriate strains occurred in each of the capsule serotypes 1–72 and mostly showed the biochemical reactions of saprophytic *Klebsiella aerogenes*. The fimbriae were clearly distinguishable from the capsules and occurred also in non-capsulate mutants. Most fimbriate strains showed evidence of varying reversibly between a fimbriate and a non-fimbriate phase. The proportion of bacilli with fimbriae was greatest in broth cultures and was decreased, though never eliminated, by serial cultivation on nutrient agar. All 29 permanently non-fimbriate strains belonged to serotypes 1–6 and showed biochemical reactions common in pathogenic *K. pneumoniae*, *K. ozaenae* and *K. rhinoscleromatis* strains (anaerogenic, methyl-red positive, non-citrate utilizing or non-lactose fermenting). They differed from the fimbriate strains in growing less abundantly and usually in failing to form a pellicle on broth.

All the fimbriate strains, but none of the non-fimbriate, showed one or other or both of two kinds of adhesive property: one attributed to an 'MS adhesin', susceptible to inhibition by D-mannose and associated with a thick variety of fimbriae; the other due to an 'MR adhesin', resistant to mannose and associated with thinner fimbriae. Bacilli with MS adhesin rapidly adhered to red blood cells of the guinea-pig and other animals, except the ox, to leucocytes and to intestinal epithelial cells, including those of ox, to *Candida albicans* cells, to the mycelium of *Aspergillus niger* and other moulds, and to plant root-hairs. Bacilli with only the MR adhesin did not adhere to untreated red cells, leucocytes or 'smooth' yeasts, but adhered rapidly to ox and other red cells treated with tannic acid, red-cell stromata heated at 70° or 100°, fungus mycelium and plant root-hairs. Bacilli of many fimbriate strains and one non-capsulate non-fimbriate strain adhered to glass and cellulose.

Bacterial fimbriae are filamentous appendages which are smaller and more numerous than flagella, and demonstrable only by electron microscopy (Anderson, 1949; Houwink & van Iterson, 1950). Since Anderson interpreted fimbriae as strands of dried capsular substance, attention was given in the present study to establishing the morphological distinction between fimbriae and capsules, and the presence of fimbriae in non-capsulate mutants. Fimbriae occur widely among the Enterobacteriaceae, in a majority of strains of *Escherichia coli* (Duguid, Smith, Dempster & Edmunds, 1955), *Aerobacter cloacae* (Constable, 1956), *Shigella flexneri* (Duguid & Gillies, 1957) and Salmonella, Klebsiella, Proteus and Serratia (Duguid & Gillies, 1958). They are thought to act as organs of attachment, since most fimbriate strains were found to have the power of adhering to animal and fungal cells, and thus of causing haemagglutination when mixed with red blood cells. Excepting Proteus, most fimbriate bacilli have shown the same kind of adhesive property, acting strongly with guinea-pig, fowl and many other species of red cells, weakly with human and sheep cells, and scarcely at all with ox cells. This adhesive activity is almost completely inhibited by a small concentration of D-mannose or α-methylmannoside (Duguid & Gillies, 1957), and the responsible agent is therefore termed the *mannose-sensitive* (MS) *fimbrial haemagglutinin* or *adhesin*. The alternative name 'adhesin' is introduced because the fimbriae adhere to many substrates other than red cells. A minority of fimbriate Klebsiella strains were found by Duguid & Gillies (1958) to lack adhesive affinity for all species of red cells tested. In the present study other substrates were found for which these strains are adhesive, including mould mycelium and tannic-acid treated red cells. This adhesive activity is unaffected by mannose and the responsible agent is therefore termed the *mannose-resistant* (MR) *fimbrial haemagglutinin* or *adhesin*.

METHODS

Organisms. I examined 154 non-motile strains representing the Klebsiella capsule serotypes 1–72 established by Julianelle and others (see Edwards & Fife, 1952; Edmunds, 1954; Edwards & Ewing, 1955). Seventy strains in types 1–6 and a strain each of types 18 and 36 were obtained from the National Collection of Type Cultures, London; these are designated by the Collection number prefixed by the letter N (e.g. N. 5054). The type strains of types 7–63 and 70–72 were obtained from Dr P. R. Edwards and those of types 64–69 from Dr P. N. Edmunds; they are designated K. 7, K. 8, ..., K. 72. A further type-5 and four type-38 strains (E. 5, etc.) were obtained from Dr Edwards and the remaining 11 strains (A. 1, A. 3, etc., all of type 54) were isolated in Edinburgh from faeces and other sources (Duguid, 1951; Wilkinson, Duguid & Edmunds, 1954). The strains were maintained as stab cultures in nutrient agar. They were plated on nutrient agar and a trace of material from three typical colonies was taken as the starting inoculum for each test series of cultures.

Cultures were grown serially for 48 hr. periods at 37° in 10 ml. nutrient broth in ½ in. diameter test tubes plugged with cotton wool; they were incubated aerobically without agitation or artificial aeration. The broth contained (% (w/v)): 1, meat extract ('Oxoid' Lab Lemco); 1, peptone ('Difco' Bacto-peptone); 0·5, NaCl; its pH value was 7·3. In many repeat experiments which gave similar results, the peptone used was 'Oxoid' Bacteriological Peptone. Surface cultures were grown on well-dried plates of nutrient agar incorporating 1·2 % (w/v) of Davis New Zealand agar in the broth. For growth measurement experiments, 20 ml. agar medium was dispensed in a 4 in. diameter Petri dish; this was inoculated uniformly over its whole surface and incubated with the lid raised about 1 mm. to ensure free aeration and dry surface conditions. Large capsules were obtained on an excess-sugar medium (agar with 1 % (w/v) glucose or lactose, 0·1 % (w/v) peptone, inorganic salts and 1 % (w/v) phosphate; adjusted to pH 7·3; Duguid, 1948).

Measurements of growth were made on the broth cultures and on the bacilli harvested from agar plates into a volume of saline solution equal to the agar. The turbidity of a suitable dilution of the broth or suspension was measured with a 'Spekker' photoelectric absorptiometer and the bacilli were counted microscopically in a counting chamber.

Haemagglutination tests were made with untreated guinea-pig red cells and 'tanned' ox red cells. Guinea-pig cells from freshly drawn citrated blood were twice washed and then prepared as a 3% (v/v) suspension in saline solution (0·85%, w/v, NaCl). A drop of this was mixed on a white tile with an equal drop of suspension containing about 10^{10}–10^{11} bacilli/ml., prepared by centrifuging a broth culture, decanting the supernatant liquid and mixing the deposit with the residual liquid. Mixing on the tile was continued at room temperature during 15 min. on a rocking machine which tilted to and fro 50 times/min. Coarse haemagglutination was often seen within 1 min., but with weakly active cultures only a fine granularity appeared after 5–10 min. (Gillies & Duguid, 1958, fig. 2).

For the test with *tanned ox red cells*, washed red cells were treated with 0·003% (w/v) tannic acid for 10 min. at 37° by the method of Boyden (1951) and then twice washed before making up to a 3% (v/v) suspension in saline. These cells showed a beginning of agglutination while in the tannic acid but, after washing, gave a suspension which was not autoagglutinable during continuous mixing for 15 min. on the tile.

The *mannose sensitivity* of each reaction was observed by adding a small drop of a 2% (w/v) D-mannose solution to the drop of red cells before adding the bacilli; the final mannose concentration (*c.* 0·5%) completely prevented MS haemagglutination.

The *haemagglutinating power* (HP) of a culture was measured by making tile tests with bacillary suspensions of different concentration and finding the weakest suspension that would give a visible reaction within 10 min. (Duguid & Gillies, 1957). The HP was taken as 10^{11} divided by the minimal agglutinating number of bacilli/ml. in the final mixture with red cells; this number was estimated from a prior turbidity measurement of the bacillary suspension, assuming that a reading of 1·0 was equivalent to 10^9 bacilli/ml. (it was equivalent to $0{\cdot}5$–$2{\cdot}0 \times 10^9$/ml. in most cultures that were counted).

Adhesion tests. The adhesion of bacilli to red cells was observed microscopically in a wet film of part of the haemagglutination test mixture (Duguid *et al.* 1955). Other cells, fungus mycelium and cellulose fibres were tested by mixing them with about 10^{10} bacilli/ml. in a drop of saline solution (0·85%, w/v, NaCl) on a microscope slide. This was tilted to and fro for about 1 min., and viewed against a dark background to detect agglutination. A coverslip was then applied and the wet film examined by the phase-contrast microscope with a $\frac{1}{12}$ in. oil-immersion objective. Yeasts and fungi for these tests were grown on nutrient or Sabouraud agar for 1 day at 30° and then for a few days at 15–20°. Ox and human leucocytes were obtained from the buffy coat on centrifuged citrated blood, and guinea-pig leucocytes from the peritoneal cavity one day after injecting 25 ml. nutrient broth. Epithelial cells were scraped from the mucosal surface of guinea-pig and ox intestine (ileum and colon) which had been removed and washed with saline immediately after slaughter, and from human colon obtained at an operation. Red-cell stromata were prepared by lysing washed cells in distilled water, centrifuging and twice washing with saline. Germ-free ½–1 in. seedlings of cress and red clover were grown on nutrient agar for 2–4 days at *c.* 20° from seeds sterilized with ethanol and $HgCl_2$. The seedling was immersed for 5 min. in a saline suspension of about 10^{10} bacilli/ml., with occasional gentle shaking; it was then washed by immersion in fresh saline for 1 min. and mounted in a wet film. Cellulose fibres were obtained as ashless, chemically prepared 'Cellulose Powder' (W. and R. Balston, Ltd.). Adhesion to glass was observed on the surface of the slide when viewing a wet film of bacilli in saline. The slides were cleaned by polishing and roasting in a flame, or by soaking in chromic-sulphuric acid solution and rinsing in distilled water.

Electron-microscopical observations of fimbriae and capsules were made on gold-palladium shadowed films of bacilli killed with 0·25 % formaldehyde (Duguid & Gillies, 1957). Capsules were also observed in wet films with indian ink (Duguid, 1951). Motility was tested in semi-solid agar by the method of Tittsler & Sandholzer (1936). Biochemical reactions were tested by the methods described by Mackie & McCartney (1956); cultures in glucose peptone medium were incubated for 5 days at 30° for the methyl-red test and for 2 days at 37° for the Voges–Proskauer test; citrate utilization was tested in Koser's medium at 37° and at 30°.

RESULTS

Fimbriation among 154 *Klebsiella strains*

Shigella flexneri was found by Duguid & Gillies (1957) to vary between a fimbriate and a non-fimbriate phase, the fimbriate becoming predominant after several serial aerobic cultivations in broth and the non-fimbriate after serial cultivation on nutrient agar. In view of this, each Klebsiella strain was examined in 9–10 serial cultures grown aerobically at 37° for 48 hr. in tubes of nutrient broth. The first broth tube was inoculated from three colonies on agar and subsequent broth tubes with a small loopful from the surface of the preceding culture, including a fragment of any pellicle present. Haemagglutination tests were made on most of the ten cultures and electron-microscopical examinations on at least two, usually the second and sixth. A strain was accepted as fimbriate only when fimbriae were clearly demonstrated in two cultures. Many strains, including all initially found to be non-fimbriate or doubtfully fimbriate, were examined in a second series of 37° broth cultures started separately from the stock culture. The strains still found non-fimbriate were examined in a series of broth cultures grown at 30° and in a culture on nutrient agar.

Of the 154 strains, 125 were found to be fimbriate, including representatives of all 72 serotypes (Table 1). Five were moderately or richly fimbriate in cultures at 30°, but non-fimbriate or very poorly fimbriate at 37°; these included four type-2 strains and the type-52 strain reported as non-fimbriate by Duguid & Gillies (1958). The fimbriate strains included all of 98 which had the biochemical reactions typical of saprophytic *Klebsiella aerogenes* (methyl-red negative, Voges–Proskauer positive, citrate utilizing, aerogenic in fermenting glucose, lactose and other sugars, and non-indole-producing). Also fimbriate were 27 strains with atypical reactions: 19 with reversed methyl-red and Voges–Proskauer reactions (mostly in types 2, 3 and 5), 3 not utilizing citrate (e.g. N. 9619), 4 anaerogenic (e.g. K. 26) and 9 producing indole (e.g. N. 8167).

The 29 strains found permanently non-fimbriate occurred exclusively in serotypes 1–6. All showed some of the atypical biochemical reactions that are common in *pneumoniae*, *ozaenae* and *rhinoscleromatis* strains of Klebsiella: 25 had reversed or doubtful methyl-red and Voges-Proskauer reactions, 9 did not utilize citrate (5 in types 3 and 4), 13 were anaerogenic in fermenting

Table 1. *Fimbriae and mannose-sensitive (MS) and mannose-resistant (MR) haemagglutinins (adhesins) in* 154 Klebsiella *strains of capsule serotypes* 1–72 *in broth cultures at* 37°

+ = fimbriae or haemagglutinin present in most cultures of strain. $+^{30}$ = present in growths at 30° and absent at 37°. − = absent. ± = haemagglutinating activity never more than very weak. +, ± = activity strong in some strains and very weak in others. (±) = very weak activity in occasional cultures, none in most.

				Haemagglutinins	
Serotype	No. of strains	Example strains	Fimbriae	MS, guinea-pig cells	MR, tanned ox cells
1	8	N. 5054, N. 9618	−	−	−
	4	N. 9494, N. 9499	+	+	+
2	2	N. 7242, N. 7761	−	−	−
	12	N. 9503, N. 9621	+	+, ±	+
	4	N. 5055, N. 9620	$+^{30}$	$-, \pm^{30}$	$\pm, +^{30}$
3	3	N. 5046, N. 5047	−	−	−
	8	N. 5056, N. 9633	+	+, ±	−
4	9	N. 5050, N. 9626	−	−	−
	1	N. 9644	+	+	−
	1	N. 9646	+	±	+
	2	N. 9652, N. 9656	+	−	+
5	5	N. 5051, N. 9664	−	−	−
	2	N. 9660, N. 9663	+	+	+
	1	E. 5	+	−	+
6	2	N. 5052, N. 9666	−	−	−
	7	N. 9668, N. 9670	+	+, ±	+
18	1	K. 18	+	+	+
	1	N. 8167	+	−	+
36	1	K. 36	+	+	+
	1	N. 5939	+	−	+
38	1	K. 38	+	−	$(\pm)^{30}$
	2	E. 4324, E. 4683	+	+	+
	2	E. 2802, E. 5070	+	+	(±)
54	12	K. 54, A. 3	+	+	+
The type-strain only was examined in each of the following types:					
40, 55			+	+	−
8, 10–17, 19–25, 27, 28, 30, 32–35, 37, 39, 41–51, 53, 56–64, 72			+	+, ±	+
31, 65, 71			+	$+^{30}$	+
7, 9, 26, 29, 66–70			+	−	+
52			$+^{30}$	−	$+^{30}$

glucose, 4 did not ferment lactose (e.g. N. 5046) and others gave delayed fermentation of lactose. None of the 154 klebsiellas was motile in semi-solid agar and none showed flagella by the electron microscope.

Morphology of fimbriae and capsules

Except where obscured by large capsules or a deposit of slime, the fimbriae of Klebsiella strains with MS adhesin showed the same morphology as the fimbriae of *Escherichia coli* and *Shigella flexneri* (Pl. 1, fig. 1; Pl. 2, fig. 4; Pl. 3, fig. 7). They were similar in length, width (*c.* 0·01μ), absence of flagellar-type wave form, peritrichous distribution and number/bacillus (often 100–300, but sometimes under 50). In strains having only the MR type of adhesive activity, the fimbriae appeared to be thinner (perhaps about 0·007μ) and more pliable (Pl. 3, fig. 5). Since the apparent width of the fimbriae varied to some extent with uncontrolled minor differences in specimen-preparation and microscopy, the distinction between the two kinds of fimbriae could not be established beyond doubt. It was most convincingly apparent in cultures having both the MS and MR adhesins, where the same microscope field showed some bacilli with the thick fimbriae and others with the thin; the two kinds were not seen together on the same bacillus.

On examination of broth cultures by the wet-film indian ink method, 151 of the 154 strains were found to be capsulated, though some showed visible capsules on only a proportion of cells (e.g. N. 9494). Strains N. 9655, N. 9663 and N. 9666 appeared to be non-capsulate. In broth and on nutrient agar most strains formed small capsules, e.g. 0·2–0·5μ in radial thickness and 1·2–2·0μ in transverse diameter; a few formed much larger capsules, mainly 2–4μ in transverse diameter (e.g. all the non-fimbriate type-1 strains and the fimbriate type-4 strain N. 9644).

The capsules were less clearly demonstrable by the electron microscope due to their low solid content and great shrinkage on drying. They usually appeared as a narrow, indefinite zone of faint opacity and were recognized mainly because they obscured the sharp margins of the cell-wall fold which surrounds the shrunken bacillary protoplast (cf. Pl. 2, figs. 2 and 3). In fimbriate bacilli, they usually obscured the fimbriae for only a very short distance at their proximal ends (Pl. 1, fig. 1). In bacilli with very large capsules grown on excess-sugar medium, the fimbriae could be seen passing through the capsule and then outwards beyond it (Pl. 3, fig. 6). In some parts of most films, the fimbriae were matted together and grossly obscured by the capsular substance and slime.

Non-capsulate non-slime-forming (O) mutants were isolated from the broth cultures of 16 capsulate fimbriate strains, including N. 9499, K. 9, K. 55 and A. 3. These mutants gave non-mucoid growths on nutrient agar and excess-sugar medium, showing no trace of capsules or slime in indian ink films. The A. 3(0) mutant was proved by serological tests to lack the specific type-54 exopolysaccharide of its parent strain (Wilkinson *et al.* 1954, Table 2). All these non-capsulate mutants were found to be normally fimbriate. Their fimbriae and cell walls were seen unobscured by capsular material (Pl. 2, fig. 4).

Haemagglutinating and adhesive properties

All 154 strains were tested for haemagglutination of untreated guinea-pig red cells and tanned ox red cells, and for the susceptibility of their reactions to inhibition by mannose; tile tests were made with the centrifuged bacillary deposits of the serial broth cultures and the results are shown in Table 1. All strains were further examined in saline wet films for adhesion to the mycelium of *Aspergillus niger* and the surface of the glass slide. The 'strength' of the adhesive activity of a culture was judged from the number of bacilli which adhered to the substrate within a few minutes. Firm adhesion of the bacilli was assumed from their lack of Brownian movement and their failure to move from the site of adherence when disturbed by quick small movements of the fine-focusing adjustment. Reactions with other substrates were tested on a selection of 15–30 representative strains, including N. 5054, N. 7242, N. 7242(0), N. 9499, N. 9644, N. 9652, K. 9, K. 40, K. 55, E. 5, A. 3 and A. 3(0).

Table 2. *Adhesive affinity for various substrates of fimbriate strains with MS and MR adhesins and of non-fimbriate strains*

Results in first three columns are for richly fimbriate cultures only; those in third are for cultures with MS and MR adhesins in about equal strength. – = no adhesion, ± = very few bacilli adhering, + to + + + = increasing numbers of bacilli adhering within 5 min. period. Alternative results, e.g. –, + + +, are extremes for different cultures and strains. Rare results are shown thus (+) etc. The *mannose-sensitivity* of the reaction is shown as S = sensitive, R = resistant, SR = slightly sensitive.

Substrates	Strains with MS adhesin only	Strains with MR adhesin only	Strains with MS and MR adhesins	Non-fimbriate strains
Guinea-pig red cells	+ + + S	–	+ + + S	–
Guinea-pig red-cell stromata	+ + + S	± R	+ + + S	–
Heated guinea-pig red-cell stromata	+ + + S	+ + + R	+ + + R	–, (±)
Tanned guinea-pig red-cells	+ + + S	+ + + R	+ + + R	–, (±)
Guinea-pig leucocytes	+ + + S	–	+ + + S	–
Guinea-pig intestinal epithelium	+ + + S	+ R	+ + + SR	–
Ox red cells	–	–	–	–
Ox red-cell stromata	± S	–	± S	–
Heated ox red-cell stromata	+ S	+ + + R	+ + + R	–, (±)
Tanned ox red cells	+ S	+ + + R	+ + + R	–, (±)
Ox leucocytes	+ + + S	–	+ + + S	–
Ox intestinal epithelium	+ + + S	+ R	+ + + SR	–
'Smooth' *Candida albicans*	+ + + S	–	+ + + S	–
Heated 'smooth' *C. albicans*	+ + + S	+ R	+ + + R	–
'Rough' *C. albicans*	+ + + S	+ R	+ + + R	–
Aspergillus niger mycelium	+ + + S	+ + + R	+ + + R	–, (±)
Cress plant root hairs	+ + + S	+ + + R	+ + + R	–, (±)
Glass slide	–, + + + R	+ + +, (+) R	+ + +, (+) R	–, (+ +) R
Cellulose fibres	–, + + + R	+ + +, (+) R	+ + +, (+) R	–, (+ +) R

The 154 strains were divisible into four groups having the adhesive properties shown in the four columns of Table 2. There were: 11 fimbriate strains with MS activity only; 18 fimbriate strains with MR activity only; 96 fimbriate strains with MS and MR activities together; 29 non-fimbriate strains virtually devoid of adhesive activity. Only the 107 strains which had MS adhesin were able to agglutinate untreated guinea-pig red cells, while only the 114 strains which had MR adhesin were able to agglutinate tanned ox red cells in 0·5% (w/v) D-mannose. Both the MS and MR adhesins were active for *Aspergillus niger* mycelium and certain other substrates; cultures which had both adhesins gave mannose-resistant reactions with these substrates.

The stability of the fimbriae was similar to that found in *Shigella flexneri* by Gillies & Duguid (1958). The MS and MR haemagglutinating properties were destroyed by heating the bacilli at 90° for 30 min., but were usually little affected by heating at 60° for 30 min. or by exposure to 2% formaldehyde at 37° for 3 hr. The MR activity of a few strains (e.g. K. 9) was markedly decreased by the latter procedures. No evidence was obtained for the existence of a soluble haemagglutinin; neither MS nor MR activity could be demonstrated in bacteria-free supernatant fluids from centrifuged broth cultures.

Properties of the MS fimbrial adhesin

The red cells found most susceptible to MS agglutination were those of the guinea-pig. The haemagglutinating power (HP) of a culture for these red cells differed with the strain and degree of fimbriation (Table 3). The most strongly active (e.g. of K. 55) had an HP value of 1000–3000, giving a just visible reaction in mixtures of about 1 bacillus/2–6 red cells. The weakest (e.g. of N. 9633) had an HP value of less than 5, requiring over 100 bacilli/red cell to give a visible reaction. Highly active cultures also agglutinated fowl, toad and horse red cells fairly strongly, rabbit and human cells weakly, sheep cells very weakly and ox cells not at all (some K. 55 cultures gave a trace reaction with ox cells).

Haemagglutination was caused by a rapid firm adhesion of bacilli to the red cells. This was seen microscopically in wet films as described by Duguid *et al.* (1955). Fimbriate bacilli with large capsules (e.g. N. 9644) appeared fixed to the red-cell surface with an intervening gap of up to 2μ; addition of indian ink showed that this gap was occupied by capsule. The presence of 0·5% D-mannose in all cases inhibited the agglutination of untreated red cells. Usually inhibition was complete, but in some cultures (especially at 30°) of a few strains (e.g. of type 3) it was only partial. In the latter case, microscopical examination showed that the adhesion of bacilli had been prevented by the mannose and that the red cells were weakly agglutinated by a fine crystalline debris apparently derived from the culture. Haemagglutination was inhibited by 0·5% α-methylmannoside as effectively as by mannose, but was unaffected by 0·5% or 2·5% of D-glucose, α-methylglucoside, D-lyxose, D-galactose, D-xylose, D-raffinose, L-arabinose, L-rhamnose, maltose, lactose, sucrose, glycerol, mannitol or sorbitol.

Strains with MS adhesin but no MR adhesin. These adhered to and agglutinated the washed stromata of lysed guinea-pig red cells, and also stromata which had been heated at 70° or 100° for 5 min. They agglutinated red cells treated with 0·003% (w/v) tannic acid solution even more strongly than untreated cells, and thus often gave a definite though weak reaction with tanned ox cells. They rapidly adhered to and agglutinated guinea-pig, human and ox leucocytes (Pl. 4, fig. 8); on mixture with the buffy coat of ox blood they agglutinated the leucocytes and platelets, leaving the red cells unaffected. The MS strains showed rapid adhesion to fresh epithelial cells scraped from the mucosa of guinea-pig ileum and colon, human colon and ox ileum and colon, as described for *Shigella flexneri* by Duguid & Gillies (1957). They also adhered to and strongly agglutinated the yeast-like cells of *Candida albicans*, from two smooth-colony strains and from a rough-colony strain whose cells tended to remain attached in groups. The MS strains adhered strongly to the mycelium and conidia of various saprophytic and parasitic fungi, namely *Aspergillus niger*, *Penicillium* sp., *Rhizopus* sp., *Epicoccum* sp., *Fusarium nivale*, *Thamnidium elegans*, *Trichoderma viride*, *Phoma foveata*, *Helminthosporium avenae*, *Botrytis cinerea*, *Monosporium apiospermum*, *Trichophyton sulphureum* and *Microsporum gypseum* (see similar adhesion by MR strain, Pl. 4, fig. 10). These MS strains also adhered to the root hairs of cress and red clover seedlings (see similar adhesion by an MS and MR strain, Pl. 4, fig. 9). All these adhesive activities were largely inhibited by the presence of 0·5 % mannose.

Table 3. *Mannose-sensitive* (*MS*) *and mannose-resistant* (*MR*) *haemagglutinating powers of serial* 48 *hr. aerobic cultures at* 37° *on agar plates and in tubes of broth*

Haemagglutinating power (see methods), − = nil, ± = 2–8, + = 9–36, + + = 40–160, + + + = 180–750, + + + = 800–3000. MS power tested with guinea-pig cells and MR with tanned ox cells. The 'Agar' and 'Broth' ries were started from the same 3-colony inoculum, and the 'Broth^2' series from a different agar inoculum. The st plate of the 'Agar B' series was inoculated from the 9th broth culture of the series shown immediately above it.

		1st culture		3rd culture		5th culture		9th culture	
Strain	Medium	MS	MR	MS	MR	MS	MR	MS	MR
N. 9633	Agar	±	−	±	−	±	−	±	−
	Broth	+	−	+	−	±	−	+	−
K. 55	Agar	+	−	±	−	+	−	±	−
	Broth	+ + + +	−	+ + + +	−	+ + + +	−	+ + + +	−
	Agar B	+ + +	−	±	−	±	−	+	−
N. 9499	Agar	+ +	±	+	−	+	±	+ +	−
	Broth	+ +	+	+ + +	+ +	+ + +	+ + +	+ + + +	+ + +
	Agar B	+ + +	+ +	+	±	+	−	+	±
A. 3	Agar	±	−	±	−	+	±	±	±
	Broth	±	−	+ + +	−	+ + +	±	+ + +	±
	Agar B	+ +	+	+	±	+	−	±	±
	Broth^2	+ +	+	+ + +	+ +	+ +	+ + + +	+ +	+ + +
	Agar B^2	+ +	+ + +	+ +	+ + + +	+ +	+ + + +	+ + +	+ + + +
K. 13	Agar	±	+ + +	±	+ + +	+	+ + +	±	+ + +
	Broth	+ + + +	+ + +	+ + +	+ + + +	+ + +	+ + + +	+ + + +	+ + +
N. 9503	Agar	±	+ + +	±	+ + +	±	+ + +	±	+ + +
	Broth	±	+ + + +	±	+ + +	±	+ + + +	−	+ + + +
K. 9	Agar	−	+ + + +	−	+ + + +	−	+ + + +	−	+ + + +
	Broth	−	+ + + +	−	+ + + +	−	+ + + +	−	+ + + +

Some cultures of strains K. 40 and K. 55 showed adhesive affinity for glass and cellulose. When mounted in a saline wet film, up to 30% or more of the bacilli might become firmly attached to the surfaces of the slide and coverslip within 2–3 min., and many to cellulose fibres when these were included. This adhesive property seemed to be unrelated to the MS adhesin, since it was unaffected by mannose and was not present in many richly fimbriate cultures with strong MS activity (e.g. N. 9644). Tests made in distilled water showed that electrolyte (e.g. 0·85% NaCl) was required for adhesion to glass and for MS adhesion to *Aspergillus niger*. Mycelium and bacilli (K. 40 or K. 55) were killed by 0·5% formaldehyde and washed in several changes of distilled water before mounting together between a washed slide and coverslip. Very few bacilli adhered to the mycelium or slide during 5 min. in distilled water, but many began to adhere soon after the addition of a little salt to the edge of the film.

Non-haemolytic fimbriate strains of *Escherichia coli*, *Aerobacter cloacae*, *Shigella flexneri* and *Salmonella* have the same adhesive properties as the Klebsiella strains with MS adhesin only (Table 2, first column). Their reactions with substrates not previously tested by Duguid *et al.* (1955), Constable (1956) and Duguid & Gillies (1957, 1958) were demonstrated in the present study for a few representative strains of each species. Ten strains of *Serratia marcescens* acted as Klebsiella strains possessing MS and MR adhesins, or MR adhesin only.

Properties of the MR fimbrial adhesin

The 18 strains having only the MR adhesin neither adhered to nor agglutinated any of the following preparations: fresh red cells of the guinea-pig, fowl, toad, horse, mouse, rabbit, man, sheep and ox; unheated guinea-pig and ox red-cell stromata; guinea-pig, human and ox leucocytes; unheated 'smooth' *Candida albicans*. They did not cause haemagglutination even in tests made at 3–5° (cf. haemolytic *Escherichia coli* strains, Duguid *et al.* 1955), and showed only a scanty adhesion of bacilli to intestinal epithelial cells.

On the other hand, the MR strains rapidly adhered to and strongly agglutinated red-cell stromata which had been heated in saline at 70° or 100° for 5 min., and intact red cells which had been treated with 0·003% (w/v) tannic acid solution, irrespective of whether these had originated from ox, man, guinea-pig or other animal. Tanned ox cells were chosen for the standard test of MR activity, since they gave the most stable suspensions and were the least susceptible to MS adhesin.

All the MR strains showed strong adhesion to the mycelium and conidia of *Aspergillus niger* and other moulds (Pl. 4, fig. 10). Such strains also adhered strongly to the root hairs of cress and red clover seedlings (cf. MS and MR strain, Pl. 4, fig. 9). They adhered, usually weakly, to 'smooth' *Candida albicans* that had been heated in saline at 100° for 5 min. and to unheated cells of the 'rough' *C. albicans* strain. In saline wet films they adhered to the glass slide and coverslip, and to cellulose fibres; in most cultures this tendency was very strong and up to 50% or more of the bacilli might become attached within 1–2 min. Many MR cultures were autoagglutinable. All these adhesive activities were resistant to inhibition by mannose. They were partly dependent on the presence of electrolyte, since adhesion to fungus mycelium and glass was less when the tests were made in distilled water instead of saline.

Properties of non-fimbriate strains

All 29 non-fimbriate strains were found to lack adhesive affinity for guinea-pig red cells, tanned ox red cells, *Candida albicans*, *Aspergillus niger* mycelium and glass. At most, a rare bacillus was found adherent at a few places on the tanned cells, mould mycelium or glass. Representative strains of each serotype lacked adhesive affinity for fowl, toad, horse, mouse, rabbit, human, sheep and ox red cells, heated red-cell stromata, guinea-pig, human and ox leucocytes, intestinal epithelial cells, plant root hairs and cellulose fibres (Table 2). Exceptional results were obtained with a non-capsulate mutant derived from the non-fimbriate strain N. 7242. Its cultures were partly autoagglutinable in saline and showed a moderate adhesiveness for glass and cellulose. These properties were dependent on the presence of electrolyte. They were also found in some non-fimbriate strains of other species (e.g. *Escherichia coli* strain 55 of Duguid *et al.* 1955).

Weak mannose-resistant haemagglutination reactions were given by the concentrated bacillary deposits from a few cultures of non-fimbriate strains, especially those grown at 30°; microscopical examination revealed that these reactions occurred without adhesion of bacilli to red cells. The clumped red cells appeared interspersed with and bound together by a fine particulate debris and some bundles of long tyrosine-like crystals, presumably products of bacterial growth. When using the haemagglutination test to detect fimbriation in klebsiellas, it is thus necessary, when a weak reaction is obtained, to demonstrate microscopically that it involves bacillary adhesion. Weak non-bacillary agglutination occurred similarly with tanned red cells, red-cell stromata and *Candida albicans*.

Variation in fimbriation and haemagglutinating activity

The broth cultures of all fimbriate strains were found to contain a proportion of non-fimbriate as well as fimbriate bacilli. In most strains 50–90 % of the bacilli were fimbriate and in a few (e.g. of type 3) never more than 5–25 %. This was probably the result of frequent reversible variation between a fimbriate phase and a non-fimbriate phase, as found in *Escherichia coli* by Brinton, Buzzell & Lauffer (1954) and *Shigella flexneri* by Duguid & Gillies (1957). Cultures of a few strains (e.g. K. 9) never showed more than *c.* 10 % of non-fimbriate bacilli, and the possibility must be admitted that these were originally fimbriate, but had lost their fimbriae after dying in the culture or during preparation of the specimen.

The combined MS and MR haemagglutinating powers seemed to vary directly with the proportion of fimbriate bacilli in the culture. Observations were made on 35 strains carried in parallel through nine serial 48 hr. cultures on nutrient agar plates and nine serial 48 hr. cultures in broth, both series being started from the same 3-colony inoculum (e.g. ‘Agar’ and ‘Broth’ series, Table 3). For some strains a second series of nutrient agar cultures (‘Agar B’) was started with an inoculum from the richly-fimbriate 9th culture of the broth series. Maximal fimbriation was found to develop during the cultivation in broth. The serial cultivation on nutrient agar did not decrease the proportion of fimbriate bacilli in a few strains (e.g. K. 9), but decreased it to between 10% and 50% in most, and to as little as 1% in some (e.g. K. 55). In no case, however, did it yield a culture entirely in the non-fimbriate phase, as in *Shigella flexneri* and a few *Escherichia coli* strains (e.g. nos. 93, 108 and 208, Duguid *et al.* 1955). The klebsiellas behaved instead like the majority of *E. coli* strains.

When a poorly fimbriate nutrient agar culture was subcultivated in broth, it usually achieved moderate or maximal fimbriation in the first 48 hr. broth culture, though in some cases not until the second or third (e.g. A. 3, 'Broth' series, Table 3). When a richly fimbriate broth culture of a variable strain was subcultivated on nutrient agar, the first 48 hr. culture was moderately fimbriate and the second or third minimally fimbriate (e.g. K. 55 and N. 9499, 'Agar B' series, Table 3). Because conversion between the fimbriate and non-fimbriate phases was so rapid, the experiments did not show whether the change was directly impressed by the cultural conditions or was due to selection of mutants. The latter mechanism appears the more likely, since some evidence was obtained for its operation in *Shigella flexneri* (Duguid & Gillies, 1957). Fimbriate bacilli fixed while undergoing fission were mostly fully fimbriate at both ends. Rarely, however, one daughter cell was fimbriate and the other non-fimbriate (Pl. 3, fig. 7); this might be the result of fimbrial phase variation.

In strains capable of both MS and MR haemagglutination, these two activities could vary independently in strength and one might even disappear from a culture while the other remained. Serial cultivation in broth often brought about a large relative increase of MR activity, e.g. in 4 out of 6 experiments with strain A. 3 (cf. 'Broth^2' and 'Broth' series, Table 3). When the MR-rich A. 3 broth cultures were subcultivated on nutrient agar, they gave mucoid (capsulate) colonies which differed from the parent type in being more convex and opaque. This variant continued to yield fully fimbriate growths with strong MR activity through 15 serial cultures on nutrient agar ('Agar B^2' series, A. 3, Table 3), while the MS-rich parent form became mainly non-fimbriate ('Agar B' series). In other cases, fimbriation and MR activity were greatly diminished when an MR-rich broth culture was subcultivated on nutrient agar ('Agar B' series, N. 9499, Table 3). Non-capsulate (O) mutants of strains N. 9499 and A. 3 behaved in the same ways as their parent strains, but showed less tendency to produce the MR-rich variant.

Pellicle formation and amount of growth

Duguid & Gillies (1957) found that *Shigella flexneri* in its non-fimbriate phase did not form a surface pellicle when grown in broth, while the fimbriate-phase bacilli formed a thin membranous pellicle and gave a much greater amount of organism because of the free aeration of the bacilli in the pellicle. In Klebsiella, fimbriation appeared similarly to assist pellicle formation and thus augment growth, but complicating factors make the relationship difficult to prove. A surface pellicle was formed by most fimbriate Klebsiella strains in aerobic static broth cultures. In some cases it was thin and transparent like that of *Shigella flexneri*, but usually it was thickened by a white granular, creamy or waxy growth, or by mucoid material. Few of the non-fimbriate strains formed a complete pellicle (as N. 7242 and N. 7761), but most of them formed a 'rim' or ring of solid growth which adhered to the walls of the tube at the broth surface. This rim was usually 1–4 mm. deep and extended inwards over the broth for a similar distance. In the same way as a pellicle, the rim procured highly aerobic conditions for growth. A few non-fimbriate strains in types 4, 5 and 6 (e.g. N. 9657, N. 9659, N. 9666) usually did not form a rim. Fimbriate strains formed a narrow rim in addition to their pellicle. Neither rim nor pellicle was formed in broth cultures incubated anaerobically.

Table 4. *Mean of turbidity measurements of amount of growth of fimbriate and non-fimbriate strains in broth and on nutrient agar after* 48 *hr. at* 37°

Serotype	No. of strains	Anaerobic tube of broth	'Aerobic' tube of broth	Aerobic agar plate
	Non-fimbriate; no rim or pellicle on aerobic broth			
4, 5, 6	7	0·17	0·28	2·38
	Non-fimbriate; rim but no pellicle on aerobic broth			
1	6	0·17	0·89	2·45
4, 5, 6	11	0·16	0·58	3·03
	Non-fimbriate; rim and pellicle on aerobic broth			
1	2	0·16	1·10	2·58
2	2	0·25	1·27	3·42
	Fimbriate; rim and pellicle on aerobic broth			
1	4	0·29	1·67	3·46
2	11	0·30	1·78	3·97
4, 5, 6	15	0·29	1·44	3·67

The influence of rim and pellicle formation on the amount of growth was investigated for most strains in types 1, 2, 4, 5 and 6. Turbidity measurements were made on three cultures of each strain: (1) in a tube of broth incubated anaerobically in hydrogen; (2) in a tube of broth incubated aerobically; (3) on the growth from the whole surface of a nutrient agar plate incubated aerobically. The three cultures were inoculated from the same aerobic 48 hr. broth culture (itself inoculated from nutrient agar) and were incubated at 37° for 48 hr., by which time growth was largely complete. The averages of the 48 hr. growth measurements in different groups of strains are shown in Table 4. The non-fimbriate strains which formed neither rim nor pellicle gave in the 'aerobic' broth (whose depths remained nearly anaerobic) a growth which was little more than that in the anaerobic broth (e.g. 1·5-fold) and scarcely an eighth of that on the aerobic plate. They thus behaved in the same way as non-fimbriate *Shigella flexneri* (Duguid & Gillies, 1957, Table 3). The non-fimbriate strains which formed a rim gave relatively much greater growth in 'aerobic' broth (e.g. 4 times that in anaerobic broth). The fimbriate and non-fimbriate strains which formed a pellicle gave even more (e.g. 5 times that in anaerobic broth and nearly half that on aerobic agar). The increased growth was probably due to the abundant oxygen supply enjoyed by the bacilli which composed the rim and pellicle. In cultures on agar and in anaerobic broth, where no advantage could be gained by pellicle formation, the fimbriate strains still gave greater growth than the non-fimbriate, but the difference was very much less than with the 'aerobic' broth.

DISCUSSION

Capsulated Klebsiella strains are found to be fimbriate except for a minority which correspond to pathogenic *pneumoniae*, *ozaenae* and *rhinoscleromatis* varieties. That the fimbriae and capsules are independent structures is proved by their morphological differences and the occurrence of fimbriae in non-capsulated mutants. The functions of fimbriae and capsules appear to be complementary. The highly hydrophilic, negatively charged capsule has a repellent and dispersive action, helping to prevent autoagglutination of the bacilli and their adhesion to external solids, and perhaps helping to repel phagocytes, protozoal predators and bacteriophage (Wilkinson, 1958). The fimbriae, on the other hand, confer adhesive properties on the bacilli; they pass out beyond the capsule and can fasten firmly to a variety of solid substrates. Most fimbriate Klebsiella strains seem capable of reversible variation between a fimbriate and a non-fimbriate phase. This variability may have its value in ensuring that part of a bacillary population has adhesive properties and part dispersive properties.

With one doubtful exception (K. 38), all the fimbriate strains possessed one or both of the adhesive properties attributed, respectively, to the MS and MR adhesins, while all the non-fimbriate strains lacked these properties. Many fimbriate cultures also showed a third adhesive property, effective for glass and cellulose, but this was not necessarily dependent on fimbriation, since it occurred to some extent in certain non-capsulate non-fimbriate cultures. It perhaps depended on a hydrophobic state affecting either the fimbriae or the bacillary cell wall. The electrophoretic studies of Brinton *et al.* (1954) suggest that fimbriae bear a weaker negative charge than the normal bacillary wall. This would assist their approach to negatively-charged substrates.

Only the bacilli which possessed fimbriae with MS adhesin adhered to the highly hydrophilic and negatively charged surfaces of animal blood cells and 'smooth' yeast cells. The specificity of mannose in preventing this adhesion suggests that a chemical group with some relationship to mannose may serve as a specfic receptor for the MS adhesin. This must be a common group which occurs in a wide variety of animal, plant and fungal cells. The presence of MS adhesin in bacilli infecting the animal body would probably hinder their spread by fixing them to tissue cells, and might promote their phagocytosis by attaching them to leucocytes, so nullifying the anti-phagocytic action of the capsule. This would be concordant with the absence of fimbriae from the more pathogenic strains.

Possession of the MR adhesin enabled attachment to some of the substrates susceptible to the MS adhesin, such as fungus mycelium, 'rough' yeast and plant root hairs. The MR activity, however, is not merely a low grade of the MS activity; its greater effectiveness for tanned ox red cells and its resistance to mannose show that it is due to an agent of different specificity. Similar MR activity was found in ten fimbriate strains of *Serratia marcescens*, but not in fimbriate strains of *Escherichia coli*, *Aerobacter cloacae*, *Shigella flexneri* and *Salmonella* spp., which showed only the MS activities. The MR fimbrial adhesin of Klebsiella is not to be confused with the mannose-resistant haemagglutinins found in some fimbriate and non-fimbriate strains of *E. coli* (e.g. nos. 92 and 224, Duguid *et al.* 1955; Duguid & Gillies, 1957). The latter can adhere to and agglutinate the fresh untreated red cells of one or a few species (e.g. man, sheep or ox), reacting strongly at 3–5° and 'eluting' at 20–40°. The klebsiellas do not elute on warming.

The role of fimbriae in saprophytic and commensal bacilli may be to hold them on the surfaces of dead or living animal and plant tissues, such as intestinal epithelium and root hairs, where nutrients become available by lysis, leakage or excretion. Adhesion to the surfaces of mineral solids may assist uptake of nutrient solutes which are concentrated there by adsorption. When the habitat is a stagnant liquid, advantage may accrue from the action of fimbriae in promoting growth as a surface pellicle with better access to atmospheric oxygen.

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EXPLANATION OF PLATES

Except where otherwise stated the bacilli were taken from 48 hr. cultures in broth at 37°. Figs. 1–7 are dried films shadow-cast with gold-palladium at 15°, shown by the electron microscope (75 kV). Figs. 8–10 are unstained wet films shown by phase-contrast microscope.

Plate 1

Fig. 1. Klebsiella type 54 (strain A. 3); bacillus with fimbriae and a medium-sized capsule. The capsule is much shrunken by drying and barely visible; it partially obscures the margins of the cell wall and the proximal parts of the fimbriae. (×40,000.)

Plate 2

Fig. 2. Klebsiella type 3 (N. 5047); non-fimbriate bacillus possessing a very large capsule which obscures the outlines of the cell wall. (×27,000.)

Fig. 3. *Shigella flexneri* type 2a; non-fimbriate non-capsulate bacillus showing the outlines of its cell wall unobscured; cf. fig. 2. (×30,000.)

Fig. 4. Non-capsulate mutant of a fimbriate Klebsiella type-54 strain. Note the absence of any obscuring of the cell wall and proximal parts of the fimbriae; cf. fig. 1. (×40,000.)

Plate 3

Fig. 5. Bacillus from a 48 hr. agar culture of a mutant of non-capsulate strain A. 3(O) possessing only MR adhesive activity. It bears scanty fimbriae of the very thin, pliable type associated with MR activity. (×40,000.)

Fig. 6. Klebsiella type 54 (A. 3); bacillus with fimbriae and a very large capsule, from a 72 hr. culture on phosphate-buffered 'excess-sugar' agar medium containing 1 % glucose and 0·1 % peptone. Note the fimbriae passing out through and beyond the capsule. (×28,000.)

Fig. 7. Klebsiella type 2 (N. 9502); bacillus undergoing fission; one daughter cell is fimbriate and the other non-fimbriate. (×25,000.)

Plate 4

Fig. 8. Klebsiella type 55 (K. 55); fimbriate bacilli with MS adhesin adhering to and agglutinating guinea-pig leucocytes in wet film. About 25 adherent bacilli are seen; many more were adherent in other focal planes. (×2200.)

Fig. 9. Klebsiella type 57 (K. 57); fimbriate bacilli with MS and MR adhesins adhering to tip and sides of a root hair of a cress seedling. The wet film was prepared after 5 min. exposure of the seedling to the bacilli. (×1300.)

Fig. 10. Numerous Klebsiella type 26 (K. 26) fimbriate bacilli with MR adhesin adhering to hyphae of *Monosporium apiospermum*. The few non-adherent bacilli in the wet film give very blurred images due to Brownian movement. (×2000.)

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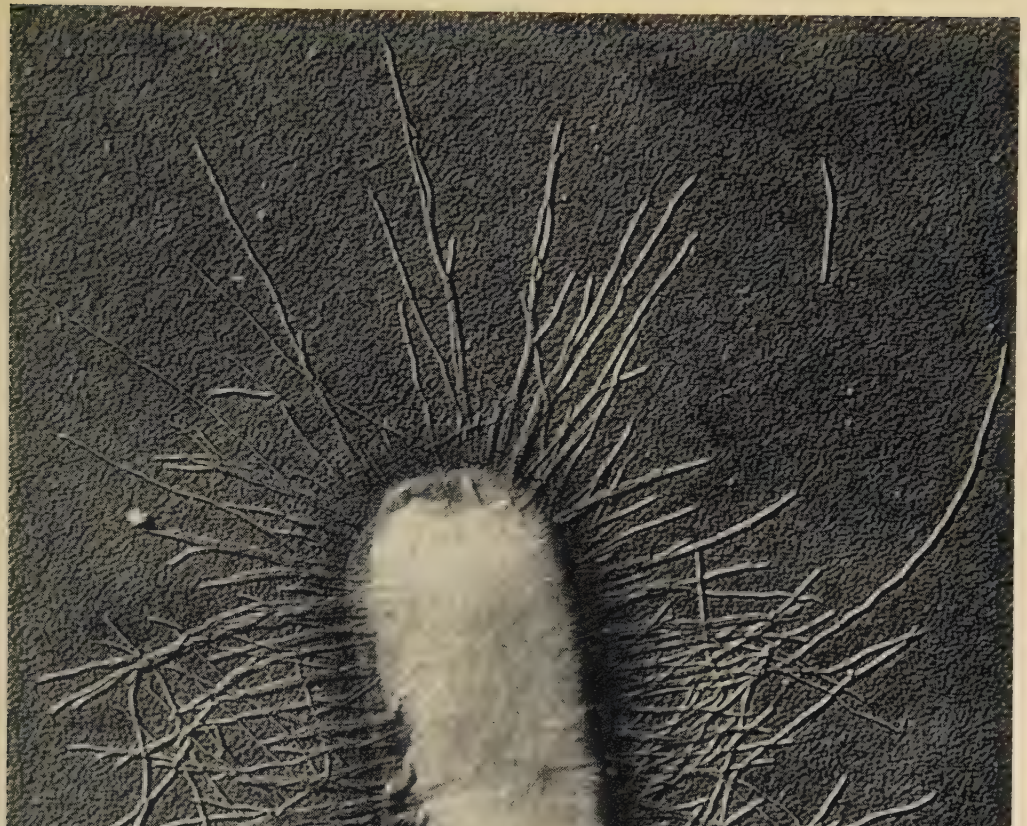
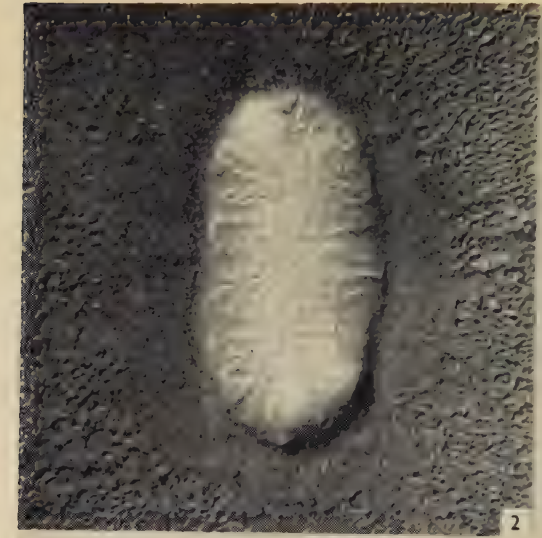


J. P. DUGUID—FIMBRIAE AND ADHESIVE PROPERTIES. PLATE 1

(*Facing p.* 286)



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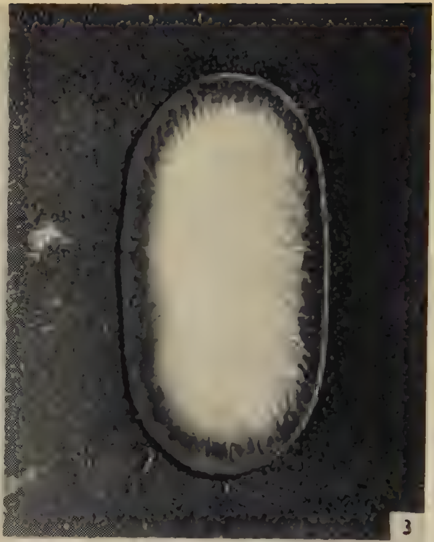

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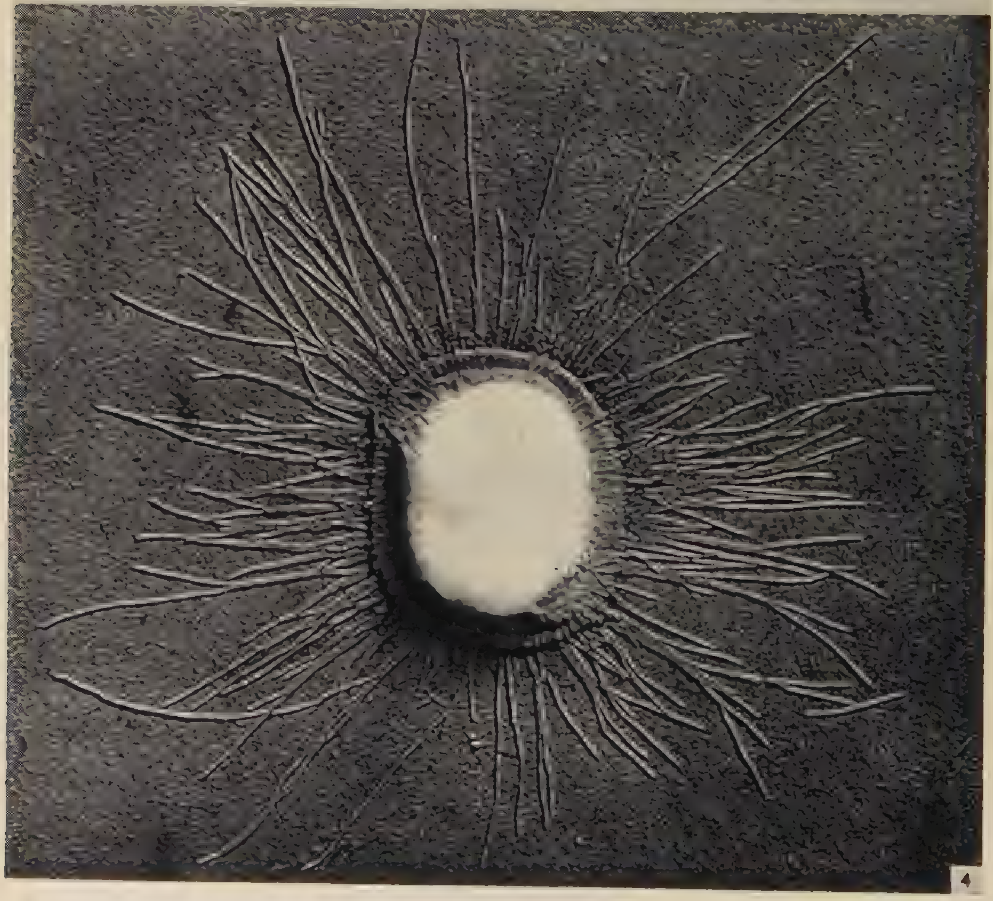

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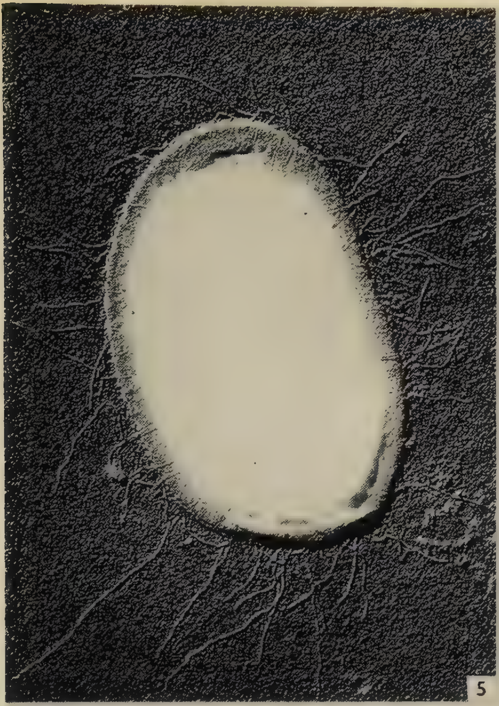
J. P. Duguid—Fimbriae and adhesive properties. Plate 2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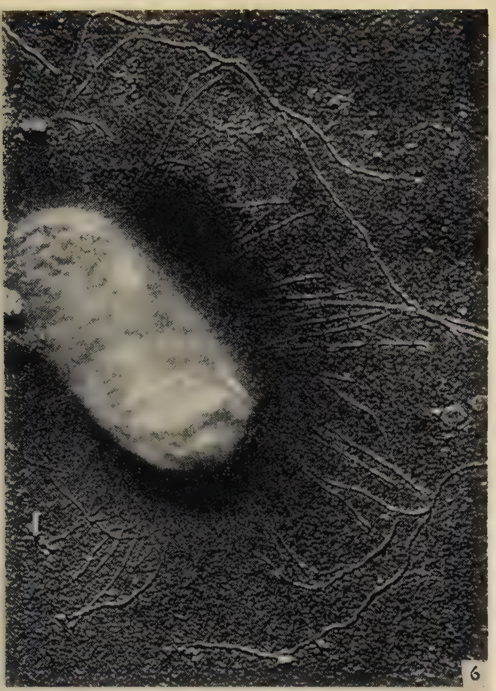
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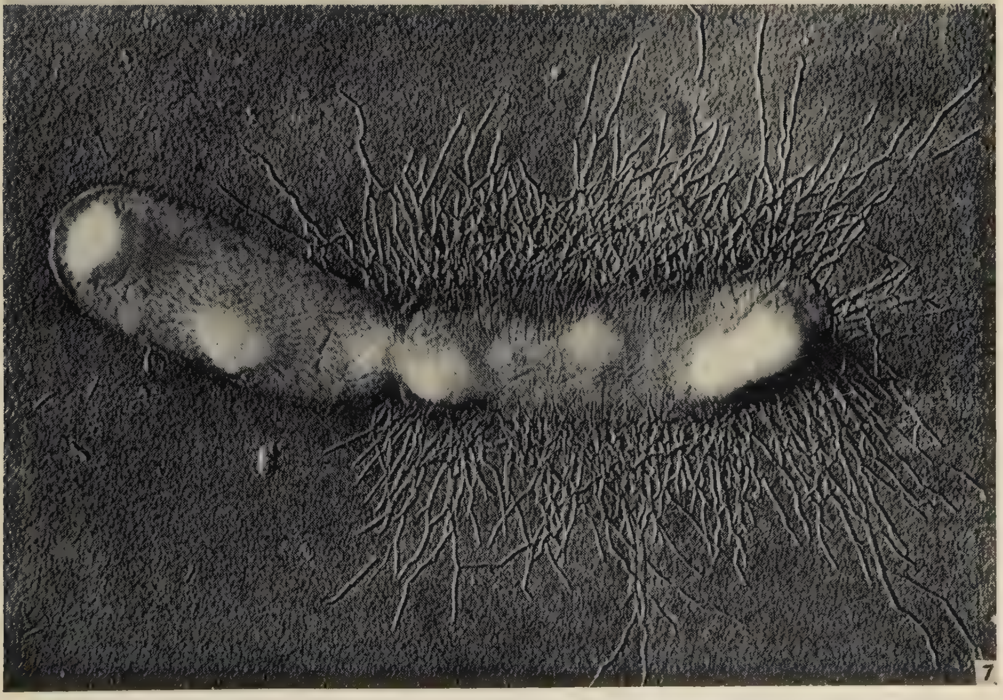
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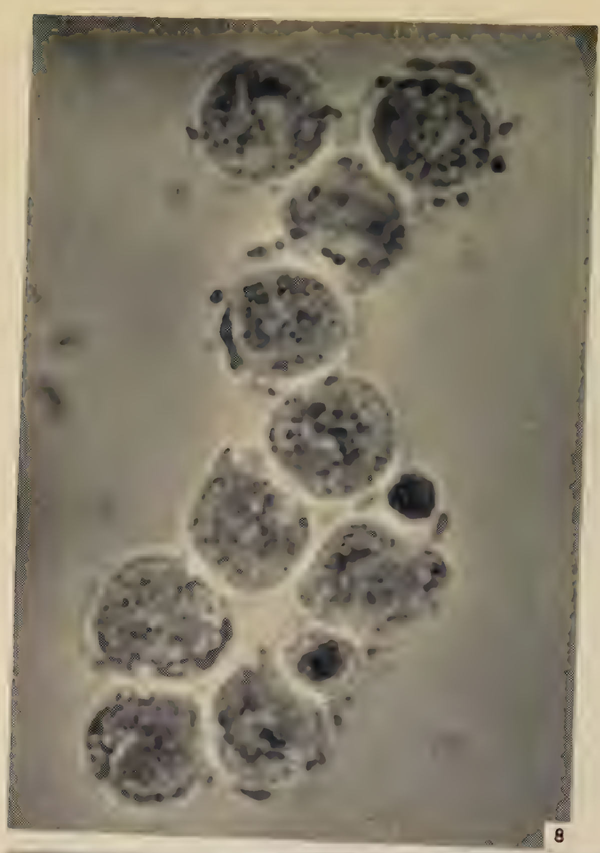
J. P. Duguid—Fimbriae and adhesive properties. Plate 3



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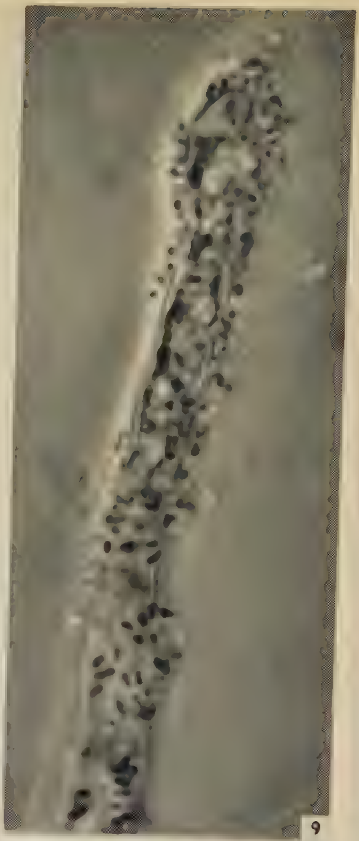

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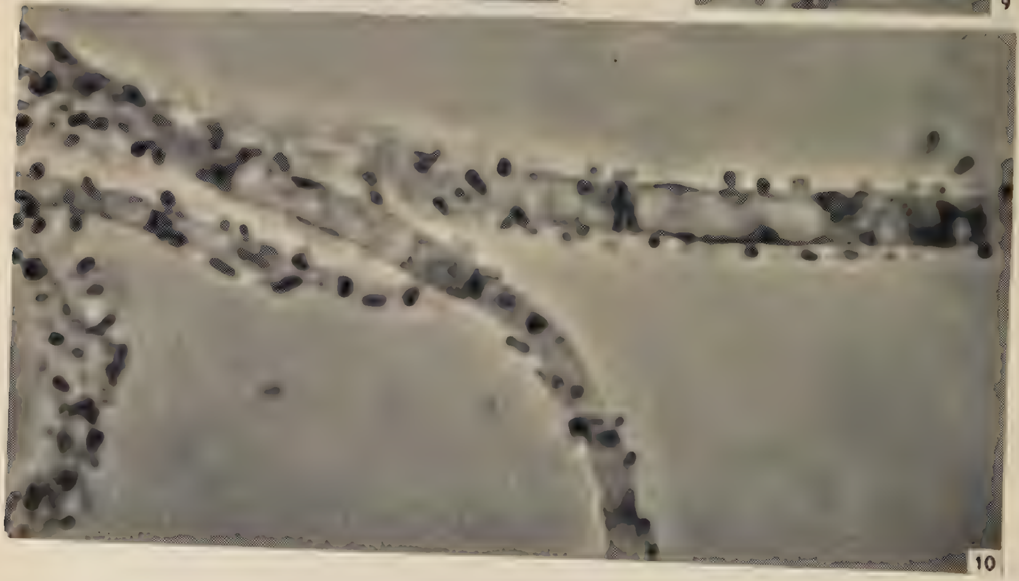

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J. P. Duguid—Fimbriae and adhesive properties. Plate 4

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A Study of Cytoplasmic Inclusions in *Trypanosoma lewisi* and their Relationship to the Formation of Antibody

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SUMMARY: Developmental forms of *Trypanosoma lewisi* continue to increase in number when the 'crisis' (normally occurring at the 10th day) is inhibited; the inclusions which they contain also increase in number, density and refractile power. Suspensions of killed developmental forms containing multiple inclusions have greater protective power against a challenge infection than have similar suspensions of adult forms which contain a single inclusion. It is suggested that the multiple inclusions contain an antigenic factor which stimulates the production of a trypanocidal antibody and reacts with it to produce the crisis, but the single inclusions of the adult forms which survive the crisis have different antigenic properties.

The formation of antibody in the early stages of an infection of the rat *Rattus norvegicus* with *Trypanosoma lewisi* has long been recognized as a constant phenomenon, and to be related to the 'crisis' which occurs at about the 10th day of the infection, checks the rise in parasitaemia and produces an abrupt fall (Steffan, 1921). This crisis marks the end of the initial 'polymorphic' phase of the infection, leaving only 'monomorphic' trypanosomes in the blood; these survive for a period varying from several days to several months. The final elimination of this monomorphic infection is also due to the formation of an antibody, but this has been shown (Taliaferro, Pizzi & D'Alesandro, 1958) to be distinct from antibody produced in the early stages of the infection and will not be considered further in this paper.

This paper deals with the relationship of the multiple inclusions which appear in the posterior end of the dividing forms of *Trypanosoma lewisi* to the formation of antibody which occurs at the time of the crisis; the suggestion that there might be such a relationship arose from the appearance of these inclusions at the time of the crisis with *T. lewisi* and from a similar finding in the very different conditions of an infection of the rat with *T. rhodesiense* (Ormerod, 1958).

The work consisted of three experiments, details of which will be given separately in the sections on Methods and Results:

(1) A study of the effect of serum, obtained from infected rats in which a normal crisis had occurred, on the number, density and refractile power of inclusions.

(2) A similar study of inclusions in rats in which the crisis had been inhibited.

(3) A comparison between the potency of suspensions of killed develop-

mental forms containing multiple inclusions, with killed adult forms containing a single inclusion, in immunizing rats against a challenge infection with the same strain of *Trypanosoma lewisi*.

METHODS

Two strains of *Trypanosoma lewisi* were used throughout the course of this work; these are named the 'Winches' strain and R.N. 36 and have been described previously (Ormerod, 1958). Transmission of the infection was made throughout by syringe, and in all experiments hooded laboratory rats were used. Pooled serum was taken from rats after the crisis had occurred but while trypanosomes were still present in the circulation; it was stored in the freezing compartment of a domestic refrigerator.

All the morphological observations were made with the ×95 objective of a Cooke, Troughton and Sims phase-contrast microscope. Trypanosomes were partially immobilized between a glass coverslip and a film of agar (2 %, w/v) in physiological saline.

Study of inclusions after injection of serum

Six rats of *c.* 100 g. each were injected intraperitoneally with *Trypanosoma lewisi*, *c.* 1000 adult organisms were given. An infection developed which could be studied satisfactorily by the 5th day. On the 5th day two of these rats were injected intraperitoneally with 0·5 and 1 ml. respectively of the serum, and on the 6th day two rats also received similar doses. The circulating trypanosomes of these rats and of the controls were examined 30 min. from the time of injection with serum and at intervals throughout the next 3 days.

Study of inclusions after inhibiting the crisis

The spleen was removed from rats of *c.* 300 g. 6 days before the initial injection of trypanosomes. From the first day after injection of trypanosomes each animal was given a daily oral dose of sodium salicylate (5 %, w/v) in distilled water by means of a syringe and needle tipped with polythene tube introduced into the stomach; 20 mg. salicylate/100 g. was enough to inhibit the crisis and to permit an infection of such extent that trypanosomes outnumbered red blood corpuscles. In the experiment shown in Table 1 several rats which were obviously infected with *Bartonella muris* died early in the infection. Two, which did not show infection with *B. muris*, survived for 23 days; they became lethargic, cyanosed and developed gangrene of the tail. In another experiment the rats were killed at 16 days without any crisis having been observed.

Immunizing properties of developmental and adult trypanosomes

Large numbers of trypanosomes in the developmental stages were obtained from infected rats in which the crisis had been inhibited by oral salicylate and splenectomy as described above. The trypanosomes were separated from blood cells by differential centrifugation at *c.* 1000 rev/min and were preserved

Table 1. *The effect of* 20 *mg. sodium salicylate*/100 *g. and splenectomy on the density of infection, the number and the refractile power of inclusions in* Trypanosoma lewisi.

No. of Rat	Procedure	Day 9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	Splenectomy and salicylate	−	−	++	++ . . .	++ . . .	++ . . .	++ . . .	++ . . .	++ . . .	++ . . .	++ . . .	++ : : :	+++ : : :	+++ : : :	Died
2		+	+	++	++ . . .	++ . . .	++ . . .	died								
3		+	+	++ . . .	++ . . .	++ . . .	++ . . .	++ . . .	++ . . .	++ . . .	++ . . .	++ : : :	++ : : :	+++ : : :	+++ : : :	Killed
4		−	Died													
5	Splenectomy only	−	−	++	++ . . .	++ . . .	++ . . .	++ . . .	++	+	+	+	+	+	+	Killed
6		+	−	++	++ . . .	++ . . .	++ . . .	++ . . .	++	+	+	+	+	+	+	Killed
7		Died +														
8		+	+	++	++ . . .	++ . . .	++ . . .	++ . . .	++	++	++	+	+	+	+	Killed
9	Salicylate only	−	−	−	+	+	+	+	++	++	++	++ . . .	++	++	++	Killed
10		+	+	++ . . .	++ . . .	++ . . .	++ . . .	++	++	++	++	+	+	+	++	Killed
11		Died day 7														
12		−	+	−	−	+	+	++	++	++	++	+	+	+	+	Killed
13	Control	−	+	++ . . .	++ . . .	++ . . .	++ . . .	+	++	+	+	+	+	+	+	Killed
14		−	−	++ . . .	++ . . .	+	+ . . .	+	+	+	+	+	+	+	+	Killed
15		−	−	++ . . .	++ . . .	+	+ . . .	+	+	+	+	+	+	+	+	Killed

. . ., Normal multiple inclusions; : : :, large and numerous multiple inclusions; −, no trypanosomes seen in 10 fields ×95 (agar film); +, more than one trypanosome seen in 10 fields ×95 (agar film); + +, more than one trypanosome seen in 1 field ×95 (agar film); + + +, more trypanosomes than red blood corpuscles.

in 2%, w/v, formol saline. The trypanosomes in this sample were highly polymorphic as shown in Pl. 1. Adult forms were present in small proportion which could not be estimated but was likely to be of the order of 5%. Similarly, trypanosomes from a normal, uninfluenced infection were separated 4 days after the initial crisis and preserved in formol saline (this presented slightly more difficulty since the yield of trypanosomes from an infected rat in which a normal crisis had occurred was less than a tenth of the yield from a rat in which the crisis had been inhibited). This second sample contained only monomorphic 'adult' trypanosomes. This was confirmed by examining the sample itself and by frequent examination of infections at the same phase of development; in no instance were immature forms observed after the crisis had occurred. The immunizing properties of the two samples were compared by injecting each of a set of clean litter-mate rats of matched weights of *c.* 100 g., at intervals of 3 days, with 6 doses of suspension; each dose was given intraperitoneally and contained 40×10^6 killed trypanosomes. Ten days after the last dose the immunized rats, together with unimmunized controls, were given a challenge dose of 1000 adult trypanosomes of the same strain.

The result of one such experiment was assessed by counting the number of trypanosomes per $\frac{1}{6}$ in. microscopic field; this produced clear-cut results which were analysed by means of the '*t*' test (Fisher, 1950); but because of the possible inaccuracies in the method of counting, the experiment was repeated by a more precise method, fuller details of which will be published later. In this method a small drop of blood on a coverslip was applied carefully to an agar film so that the red blood corpuscles spread to form an unicellular layer, lying edge to edge; and along the edge of this 'phalanx' the organisms were counted. The result was expressed as trypanosomes per 'linear field', this being the length along the edge of the phalanx circumscribed by a microscopic field.

RESULTS

Study of inclusions after injection of serum

A study of the morphology of inclusions was made after injection of serum from infected animals (see Methods). Little change in the morphology was observed beyond a slight diminution in the number of trypanosomes as compared with controls; this diminution can be ascribed to the destruction of developmental forms by the serum, as previously described by Coventry (1930). These experiments were not further developed or assessed quantitatively since the results were negative, i.e. they had shown that the inclusions were not developed in the presence of serum.

Study of inclusions after the inhibition of the crisis

The technique of inhibiting the crisis by oral sodium salicylate (Becker & Gallagher, 1947) was compared with the use of splenectomy alone and with the two techniques combined, as shown in Table 1. The best results were obtained by using the combined techniques; these were sufficiently striking for it to be unnecessary to count the number of trypanosomes obtained in the different groups. In the group treated by combined splenectomy and salicylate, not only was the crisis inhibited but the inclusions (usually produced only at the time of the crisis) continued to develop and became numerous and dense as shown in Pl. 1; normally inclusions present in *Trypanosoma lewisi* are faint and scanty as compared with other species (cf. Ormerod, 1958), but these inclusions which developed in conditions designed to decrease or inhibit the circulation of antibody continued to develop without any apparent check.

Table 2. *A comparison of the effect of immunizing rats with suspensions of killed adult forms and of developmental forms on the extent and duration of infection*

The results of two experiments are recorded; in each, day 1 is taken as the day before trypanosomes were first seen in the blood. For definition of the term 'linear field' see text.

No. of rat material used for immunization	Day of infection										
	1	2	3	4	5	6	7	8	9	10	11
Expt. 1											
Adult forms	No. of trypanosomes/$\frac{1}{8}$ in. field										
1	0	2	3	5	10	15	15	12	6	5	0
2	0	2	4	15	20	18	16	11	9	4	0
3	0	1	4	8	15	10	7	4	0	.	.
4	0	2	4	8	15	15	15	14	10	10	0
Developmental forms											
5	0	2	5	2	0	.	.	.	.	.	.
6	0	2	6	4	1	0	.	.	.	.	.
7	0	2	5	2	0	.	.	.	.	.	.
8	0	1	2	3	6	8	4	2	0	.	.
Controls											
9	0	10	(died from bacterial infection)								
10	0	14	20+	20+	20+	20+	20+	20	19	19	18
Expt. 2											
Adult forms	No. of trypanosomes/linear field										
1	0	10·1	11·6	4·2	2·0	0	0	0	0	0	0
2	0	11·2	12·4	8·2	7·2	4·2	0	0	0	0	0
Developmental forms											
3	0	4·8	5·4	5·2	1·2	0	0	0	0	0	0
4	0	5·2	3·6	2·4	0	0	0	0	0	0	0
Control											
5	0	15·9	20·0	20+	17·0	14·0	.	.	.	.	.

Expt. 1. Statistical analysis: for height of peak $t = 7{\cdot}1$ on 6 degrees of freedom, $P < 0{\cdot}001$; for length of infection $t = 4{\cdot}0$ on 6 degrees of freedom, $P < 0{\cdot}01$.

Immunizing properties of developmental and adult trypanosomes

Table 2 and Fig. 1 show the result of immunization of rats with suspensions containing equal numbers of killed trypanosomes; the two test suspensions were derived, one from rats treated by splenectomy and with salicylate and the other from rats in which the infection had undergone a normal crisis. The result shows clearly that the suspension of developmental forms had greater protecting power against subsequent challenge than the suspension of adult forms.

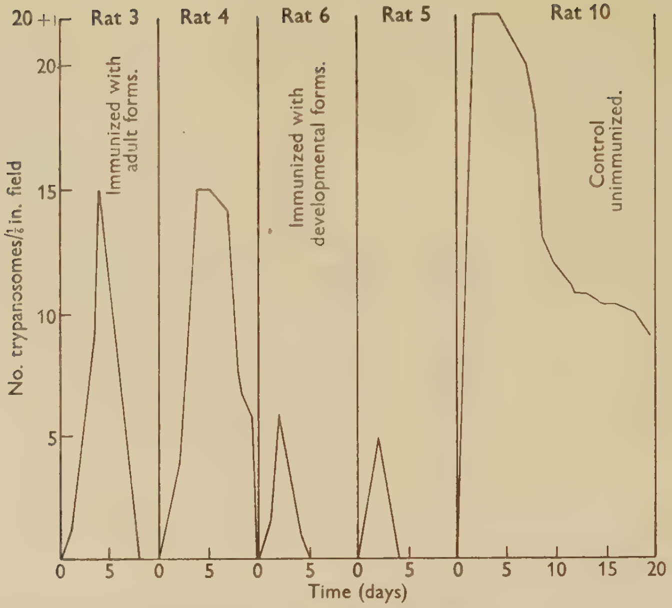

Fig. 1. The infections which resulted from a challenge dose of trypanosomes in rats which had been immunized with adult forms and developmental forms; also in an unimmunized control rat. The numbers refer to Table 2, Expt. 1.

DISCUSSION

If there is any connection between the formation of inclusions in *Trypanosoma lewisi* and antibodies which precipitate the crisis, it is not because the antibodies themselves produce the inclusions (cf. Ormerod, 1958) since inclusions develop more readily when antibody formation is inhibited. In the presence of antibody the forms which contain multiple inclusions disappear from the circulation. The inclusions are evidently produced in the natural course of the trypanosome's growth and development which is normally cut short by antibody.

These experiments show that the developmental forms are more antigenic than the adult forms of *Trypanosoma lewisi*. This provides an explanation of why in a normal infection the developmental forms are eliminated from the blood during the crisis, while the adult forms remain in circulation for a longer period until they too are eliminated from the circulation, as described in the introduction to this paper.

Reference has already been made to the work of Coventry (1930). The 'trypanolytic antibody' which she described is probably the same as the effect to which this paper refers; both are active against developmental forms and inactive against adults. Coventry's trypanolytic antibody has been accepted by Taliaferro (1932) as the direct precipitating cause of the crisis, although he continues to maintain that in addition a factor is produced which inhibits the growth of *Trypanosoma lewisi*. None of the experiments recorded here throw any light on this factor. The experiments of Thillet & Chandler (1957) are also likely to be concerned with the same type of antibody with which this paper deals, since I believe for the following reasons that their 'metabolic products' are derived from the developmental forms. These workers made a suspension of living trypanosomes from an infected rat 'before noticeable ablastin had formed' (this I take to mean that the crisis had not yet occurred and that both developmental forms and adults were present). They incubated the suspension and after a period separated the trypanosome bodies from the incubation fluid by centrifugation; rats were immunized with the incubation fluid and with the trypanosome bodies and it was found that the fluid had the greater potency for protecting rats against a challenge infection. Thillet & Chandler stated that the number of trypanosomes before and after incubation remained the same and this statement is of prime importance to their argument, although they gave no figures or limits of experimental error to support it, for it is unlikely that the developmental forms which lyse and rupture easily would survive 12 hr. incubation as readily as the more robust adults. Consequently part, at least, of the greater antigenic properties of the incubation fluid seem likely to be due to the liberation of breakdown products from developmental forms and to the relative concentration of adults among the trypanosome bodies.

Suggested mechanism of immunity

It would be unwise to attribute the immune phenomena in the early stages of *Trypanosoma lewisi* infection directly to the formation of inclusions, before demonstrating the essential fact of antigenicity of the structures themselves; but it is worth showing how simple a hypothetical mechanism of the immune process in infections with *T. lewisi* would be were one to assume as a fact the antigenicity of the inclusions. In a normal infection inclusions do not begin to develop until about the 7th day; consequently no appreciable amount of antibody would be expected to form until this time. From then on inclusions and consequent antibody would form, and the inclusion-bearing trypanosomes would be destroyed by the antibody in increasing numbers with further production of antibody until an excess of antibody over antigen was present in the blood. At this point the crisis would occur and all developmental forms be killed. The adult forms with only a single inclusion of different antigenic nature would survive as being insensitive to the circulating antibody since as has already been stated they are known to stimulate the formation of a distinct antibody. After a longer interval, however, another antibody would form against their particular antigen, and they too would disappear from the circulation.

I wish to acknowledge the receipt of a grant from the Colonial Development and Welfare Fund for technical assistance and from the University of London Central Research Fund for phase-contrast equipment. I thank Dr P. Armitage for the statistical analysis, Mr C. B. Hill and Mr P. Healey for technical assistance.

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EXPLANATION OF PLATE

Trypanosomes from a rat treated by splenectomy and sodium salicylate (Table 1, rat no. 1, day 22). The trypanosomes were partially immobilized on agar, examined by phase-contrast microscopy and drawn freehand. (For the normal appearance of developmental and adult forms of *Trypanosoma lewisi* see Ormerod, 1958.)

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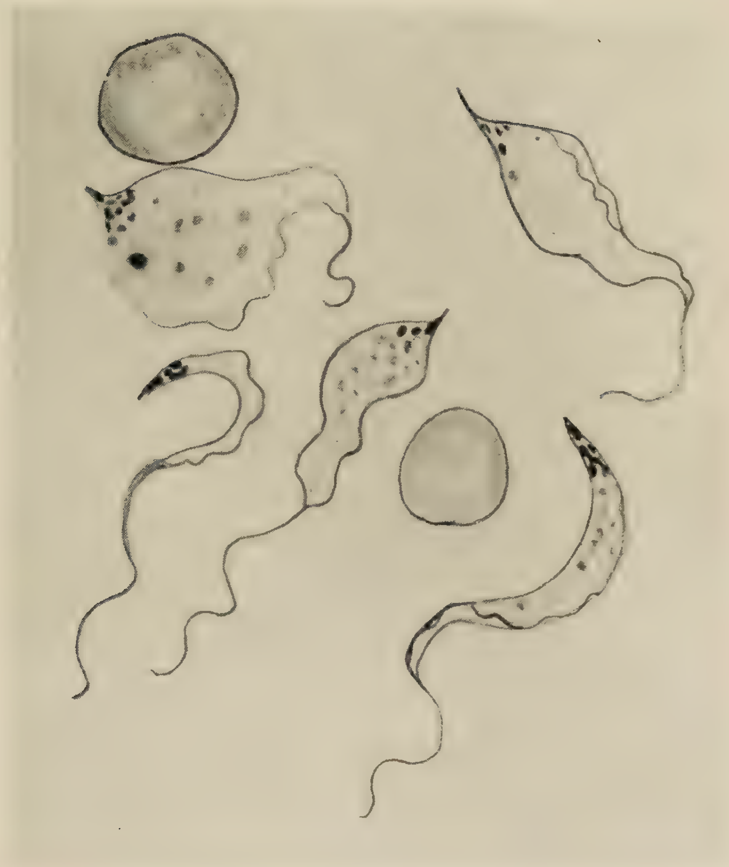

W. E. ORMEROD—CYTOPLASMIC INCLUSIONS IN *T. LEWISI*. PLATE 1

(*Facing p.* 294)

LEACH, R. H. & SCOTT, W. J. (1959). *J. gen. Microbiol.* 21, 295–307

The Influence of Rehydration on the Viability of Dried Micro-Organisms

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SUMMARY: Freeze-dried preparations of certain organisms may suffer considerable mortality during reconstitution to the wet state. For *Vibrio metchnikovi* the mortality was greatly decreased by rehydration with small volumes of water, or with various volumes of relatively concentrated solutions of some solutes. Within certain limits, slow dropwise addition of water resulted in higher viable counts than did rapid addition of the same volume of water. In preliminary tests *Mycoplasma mycoides* and two coliphages were also affected by the volume of water used for rehydration, but no significant effects were observed with nine other bacteria. Comparisons of rehydration at several temperatures showed greatest destruction at 37° for *V. metchnikovi*, but at 0° for three other bacteria. The importance of rehydration rates and of exposure to hypotonic solutions is discussed.

Although a great deal of research has been concerned with problems of retaining viability in dried micro-organisms there has been very little attention given to the rehydration stage of the process. This is somewhat surprising since, whatever the method of drying, rehydration is inevitably required before viability can be assessed. The rehydration procedures actually used by earlier workers have not always been described. Some, including Greiff, Blumenthal, Chiga & Pinkerton (1954) and Heckly, Anderson & Rockenmacher (1958), simply restored the water evaporated by adding an equal amount of distilled water. On the other hand, several other fluids have been used, and in amounts which have also varied rather widely. Broth, which was recommended by the Medical Research Council (1958), was used by Stamp (1947), Proom & Hemmons (1949), Rhodes (1950) and Annear (1956). The volume of broth used by these workers has varied from less than 5 to more than 100 times the volume of the water removed. Fry & Greaves (1951) rehydrated their cultures with 100 volumes of Ringer solution, Mazur & Weston (1956) used 2 or 3 drops of a dilute detergent solution for an unspecified amount of dry matter, and Hutton, Hilmoe & Roberts (1951) used an unspecified volume of dilute tryptose solution. Wasserman & Hopkins (1957) rehydrated their preparations with dilute solutions, using *c.* 0·5 ml./mg. dry organisms.

The reasons for using the conditions selected by the above workers have, in general, not been supported by comparisons with alternative procedures. Several investigators have, however, reported results of comparisons between different procedures. Hiscox (1945) and Higginbottom (1945) both showed that the plate counts of spray-dried milk powders were increased by making the initial rehydration at 50° rather than at room temperature, although the magnitude of the effects varied somewhat with different types of powder and with different groups of bacteria. Speck & Myers (1946) showed that spray-dried skim-milk cultures of *Lactobacillus bulgaricus* gave viable counts 1·5 to 5 times greater when rehydration at 50° as compared with rehydration at 21–25°. They showed that this was true for rehydration with 10 or 100 volumes of water or of 0·5% (w/v) sodium citrate, and that the temperature of rehydration did not affect the total microscopic count. Warming after reconstitution was not a substitute for rehydration with fluid at 50°. In contrast, when the preparations had been freeze-dried, rehydration at room temperature resulted in the larger counts. Herrera, Peterson, Cooper & Peppler (1956) showed that rehydration of dried yeast with cold water caused a loss of viability and an increased leakage of solutes from the cells. These losses did not occur when warm water was used and could also be prevented by partial rehydration with water vapour before adding the cold liquid water (Sant & Peterson, 1958). The benefits of partial rehydration with water vapour have also been reported for yeast by Mitchell & Enright (1957) and for fungal spores by Haskins (1957).

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Wasserman & Hopkins (1957) reported that the viable counts of *Serratia marcescens* were increased several hundred-fold by rehydration with solutions of sodium malate or succinate. These large effects were, however, found with only one of three preparations studied. There is also some evidence that rehydration with complex media increased the recovery of Rous sarcoma virus (Carr & Harris, 1951) and of poliomyelitis virus (Kraft & Pollard, 1954).

When, as is usual, rehydration is carried out with liquids, the process is usually completed within a few seconds. The cells are, therefore, subjected to a very rapid change from the dry or near dry conditions of the solid state to the mixture of hydrated colloids and aqueous solutions which makes up the living cell. The complexity of the changes and the speed with which they occur are both matters which make it extremely difficult either to measure or to control the events which take place. Nevertheless, it was considered worthwhile to examine the extent to which variations in methods for adding water to dried cultures may actually cause important differences in viability immediately after rehydration. Such an investigation might be expected to reveal some general principles which would be a useful guide for developing procedures to cause the least possible damage to micro-organisms during rehydration. The present paper reports some of the results obtained when various rehydration procedures were applied to several test organisms.

METHODS

Organisms and preparation of cultures

Most of the experiments were carried out with a strain of *Vibrio metchnikovi* (NCTC 8443), an organism which is rather sensitive to drying. Suspensions of this organism were obtained from surface growth on brain + heart infusion agar after 18 hr. at 37°, or from brain + heart infusion liquid medium shaken for 18 hr. at 30°. After harvesting, organisms were concentrated by centrifugation and resuspended in the fluid in which the organisms were to be dried. Except where otherwise stated, this fluid was a 2·5% (w/v) solution of Difco peptone containing 0·5M-sorbitol.

Drying of suspensions

Replicate ampoules containing 0·2 ml. were dried for 4 hr. as described by Scott (1958). For a few experiments the specimens were dried in the equipment of Mellor & Ohye (to be published). After drying, the ampoules were stored at −20° over P_2O_5 *in vacuo* and from time to time a sufficient number was withdrawn to carry out a particular rehydration experiment. Precautions were taken to avoid condensation of water on the ampoules by ensuring that they had warmed to room temperature before the desiccator was opened.

Rehydration of suspensions

Rehydration was normally carried out by pipetting the required volume of test fluid directly into an ampoule. For amounts of rehydration fluid larger than 2·0 ml. it was necessary to use a Pasteur pipette, washing the dried culture as quickly as possible into the required volume held in a separate test-tube. After a further 2–5 min., the rehydrated material was further diluted in sufficient 0·15 M-NaCl to give the nearest tenfold dilution in terms of the original undried suspension. Closer control than this of the period of exposure to the initial rehydrating fluid was not attempted, preliminary experiments having indicated that changes in viability which might occur between the above times would generally be very small in relation to the gross changes occurring almost instantaneously upon rehydration. After further tenfold dilutions in 0·15 M-NaCl, viable counts were made. Saline of this approximate strength (0·15M-NaCl; a_w 0·995; a_w = thermodynamic activity of water in the solution) was shown to be a suitable diluent for viable counts of *Vibrio metchnikovi* (Marshall & Scott, 1958). Unless otherwise noted rehydration was carried out at room temperature (18–25°).

Viable counts

For *Vibrio metchnikovi* viable counts were performed by the technique of Miles & Misra (1938) or by the pour-plate technique with nutrient agar adjusted as described by Scott (1953) to a_w 0·990 with a salt mixture with the ratio $NaCl:KCl:Na_2SO_4:MgCl_2::5:3:1:1$ mole. Good agreement between counts from successive dilutions was obtained with this medium, which was similar in composition to those reported by Marshall & Scott (1958) to give the greatest plate count for *V. metchnikovi*. These workers found that the plate count of this organism was remarkably sensitive to variations in the ionic and osmotic properties of the medium used. Colonies were counted after 18 hr. at 37° for the Miles & Misra method, and after 2–3 days at 37° for the pour-plate method.

RESULTS

Experiments with Vibrio metchnikovi

Rehydration with liquid water. The effects of reconstituting replicate ampoules of dried cultures of *Vibrio metchnikovi* with different volumes of water are shown in Fig. 1. Each ampoule before drying held 0·2 ml. suspension, containing about 10^9 viable organisms/ml., and with a dry-matter content of *c.* 25 mg./ampoule, depending on the composition of the suspending fluid. Increasing the amount of water used for rehydration from 0·25 to 16 ml. caused the viable counts to decrease by factors of a hundred to more than a thousand (Fig. 1). Similar results were obtained with cultures dried in different suspending fluids and by different methods. With one of the suspensions, rehydration with less than 0·25 ml. water also resulted in poorer recovery.

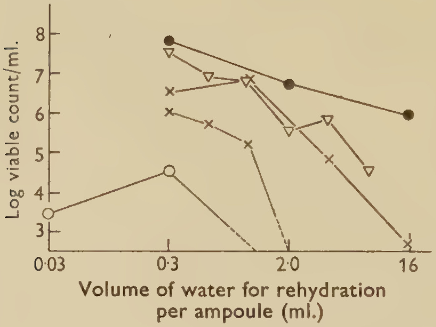

Fig. 1

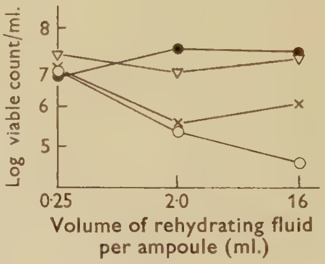

Fig. 2

Fig. 1. Survival of dried *Vibrio metchnikovi* as a function of the volume of water used for rehydration. Viable counts from 0·2 ml. samples of cultures dried in various suspending fluids and reconstituted with varied volumes of water. Suspending fluids: ×, 0·5M-glucose in papain digest broth; ▽, 0·5M-sorbitol in 2·5% peptone; ○, 0·5M-sucrose in 2·5% peptone (all dried by standard method); ●, 0·5M-sorbitol in 2·5% peptone (dried by alternative method).

Fig. 2. Survival of dried *Vibrio metchnikovi* as a function of the volume of water or other fluids used in rehydration. Viable counts from 0·2 ml. samples of a culture, dried in 0·5M-glucose in papain digest broth, and reconstituted with varied volumes of: ●, 0·5M-glucose in papain digest broth; ▽, papain digest broth; ×, 0·5M-glucose; ○, distilled water.

Rehydration with aqueous solutions. When replicate ampoules were rehydrated with different volumes of certain aqueous solutions the deleterious effect of using larger volumes was no longer observed. Results of a typical experiment are shown in Fig. 2 from which it may be seen that rehydration in papain digest broth with or without 0·5M-glucose gave viable counts which were substantially independent of the volume of fluid used. On the other hand, for 0·5M-glucose and for water itself, the viable counts were decreased when either 2 or 16 ml. of fluid were used. All four rehydration fluids gave similar counts when only 0·25 ml. was used. The effects of rehydration with different volumes, therefore, differed appreciably for different fluids. In an attempt to find some explanation for these differences the effects of the nature and concentration of the solute were studied in experiments in which the volume of fluid was maintained at 2·0 ml.

Some results for experiments of this type are shown in Fig. 3, from which it may be seen that rehydration in five types of solution was tested at each of several concentrations. As two of the solutions were mixtures and one of these consisted of a complex mixture of solutes, the concentrations have been expressed on the a_w scale (25°) using the data of Scott (1953) for the mixtures, of Robinson & Stokes (1955) for sodium sulphate and sucrose, and of Scatchard, Hamer & Wood (1938) for glycerol. For each of the materials other than glycerol there was evidence of an optimum concentration at which the viable count of the rehydrated bacteria was greatest. Whereas this optimum was at *c.* 0·985 a_w for sucrose solutions, it was 0·98 a_w for sodium sulphate, close to 0·97 a_w for brain + heart broth and *c.* 0·96 a_w for the mixed salts. For the glycerol solutions an optimum concentration was not detected, the greatest viability being obtained by using the most concentrated solution. For 0·92 a_w at 25° the glycerol is 4·44 molal or *c.* 29 %. It may be noted that when solutions of 0·92 a_w were used for rehydration, glycerol was the best of the substances tested, whereas in relatively dilute solution it gave very much smaller recoveries of viable bacteria than the other substances used. It may also be seen that the maximum recovery after rehydration in brain + heart infusion was more than a million times greater than when water was used. Results comparable to those shown in Fig. 3 were also obtained with other substances. For instance, rehydration with glucose solutions gave the greatest count when the glucose solution was of a_w 0·97. For glycine the optimum was at *c.* 0·98 a_w, and for magnesium chloride at 0·985 a_w. For these three compounds, however, the maximum counts were between only 0·2 and 2·0 % of the greatest value obtained after rehydration in concentrated brain + heart broth. Rehydration in arabinose solutions gave poor recoveries of viable organisms; with urea solutions very few living organisms were detected at all concentrations up to the maximum tested (0·92 a_w; 5·4 molal).

In view of these results it was of interest to compare the effect of the same range of concentrations of solutes on the viability of undried organisms, of organisms previously dried but already reconstituted before treatment, and of dry organisms. Samples (0·2 ml.) of a single suspension of *Vibrio metchnikovi* were therefore treated in three different ways. First, individual samples were transferred into 20 ml. of each of the test solutions, and after 2 min. diluted in 0·15 M-NaCl for viable counts. The remaining 0·2 ml. samples of suspension were freeze-dried and half of these were rehydrated directly in 20 ml. of each of the various solute concentrations, then after 2 min. diluted in saline and plated. The other samples were first reconstituted to their original (0·2 ml.) volumes with distilled water, and then treated in the same manner as the undried suspensions. Separate experiments of this kind, each at least in duplicate, were carried out with sodium chloride, sucrose and concentrated brain + heart broth at each of eight concentrations giving a_w values between 0·999 and 0·90. As the results with these three materials were very similar, only those for sodium chloride solutions are recorded (Fig. 4).

The organisms which had not been dried proved to be rather more resistant to exposure to dilute solutions, dilution in water causing a mortality of about 90 % under the conditions of the experiments. For dried organisms immediately transferred to 20 ml. of water the apparent mortality was *c.* 99·99 %, and *c.* 99·9 % when the dried organisms had first been rehydrated with 0·2 ml. water. The principal conclusion from these experiments is that the mortality caused by the rehydration of *Vibrio metchnikovi* in large volumes of water or dilute solutions is largely a consequence of exposing the freshly rehydrated organisms to dilute solutions. As previous rehydration in a small volume of water decreased, but by no means abolished, the mortality occasioned by exposing the organisms to dilute solutions, it follows that the events during the early stages of rehydration are of some importance. This conclusion is also supported by the greater viable counts obtained by direct rehydration with 20 ml. of these solutions when the a_w value was between *c.* 0·96 and 0·99.

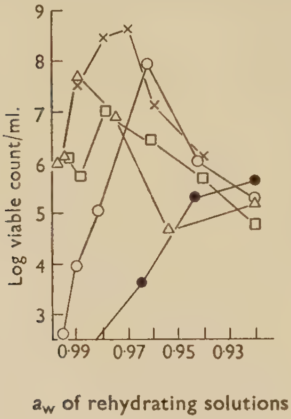

Fig. 3

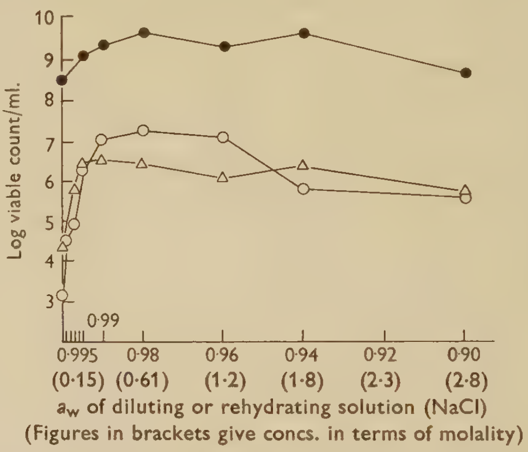

Fig. 4

Fig. 3. Survival of *Vibrio metchnikovi* as a function of the concentration of the rehydrating solution. Viable counts from 0·2 ml. samples of a culture dried in 0·2 M-sorbitol in 2·5 % peptone, and reconstituted with a fixed volume (2·0 ml.) of solutions of several solutes at different a_w values. Solutes: ○, mixed electrolytes solution ($NaCl:KCl:Na_2SO_4:MgCl_2$ in molecular ratio 5:3:1:1); △, sucrose; ●, glycerol; ×, brain + heart broth; □, Na_2SO_4.

Fig. 4. Survival of undried, dried, and reconstituted *Vibrio metchnikovi* suspensions following transfer to NaCl solutions of different a_w values. Mean viable counts from duplicate 0·2 ml. samples of a suspension in 0·5 M-sorbitol in 2·5 % peptone, transferred to 20 ml. of NaCl solutions after various preliminary treatments: ●, no preliminary treatment—undried suspension diluted directly in NaCl solution; ○, freeze-dried suspension—NaCl solution added to dry cells; △, freeze-dried suspension—reconstituted first in 0·2 ml. water, then diluted in NaCl solution.

In view of the considerable differences in the performance of different types of solutions (Fig. 3) some comparative tests were made with a series of six polyhydric alcohols, each compound being tested at six concentrations between 0·25 and 5·0 molal. Three such experiments were made, each showing similar trends; the combined results are summarized in Fig. 5 from which considerable differences in performance between solutes of differing molecular weight are evident. For the two hexitols the best recovery was obtained after rehydration in 1·0 molal solutions, which resulted in counts more than 100 times greater than those given by rehydration in water alone. For the 4- and 5-carbon compounds, erythritol and ribitol, the optimum concentrations were rather higher, being between 1–2 and 2–3 molal, respectively, and the maximum counts for these solutes were only 25–30 times better than for the water control treatments. With the smallest molecules (ethyleneglycol, glycerol) none of the concentrations tested was superior to water alone, the higher concentrations of glycerol being less effective than in the experiment shown in Fig. 8.

Although the differences associated with the different molecular weights of the solutes are not explained by these experiments, it is conceivable that the effects were at least partly connected with differences in the rates at which water entered the cells. During rehydration molecules which enter the cell more slowly than water molecules will temporarily accumulate at the external boundary of the cell, and there compete for water molecules as they enter the cell. Gradients so generated in the external solution would, of course, disappear as rehydration approached completion. The probability of obtaining a local solute concentration sufficient to cause an appreciable decrease in the

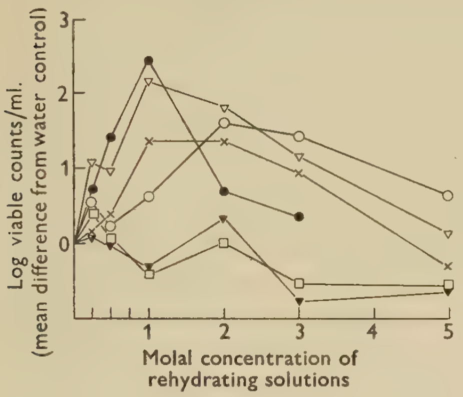

Fig. 5

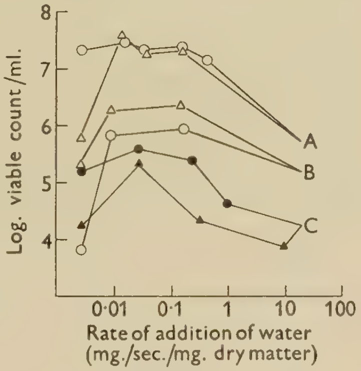

Fig. 6

Fig. 5. Survival of dried *Vibrio metchnikovi* as a function of the concentration of the rehydrating solution, for solutes of increasing molecular weight. Pooled results of three separate experiments. Viable recovery expressed as the average deviation from the mean of viable counts given by distilled water, after reconstitution of 0·1 ml. samples of suspensions, dried in 0·5 M-sorbitol in 2·5 % peptone, with a fixed amount (1·0 ml.) of varied concentrations of a series of polyols: ▽, sorbitol; ●, mannitol; ×, ribitol; ○, erythritol; □, glycerol; ▼, ethylene glycol.

Fig. 6. Survival of dried *Vibrio metchnikovi* as a function of the rate of rehydration. Viable counts from three experiments, A, B and C, in which 0·2 ml. samples of culture dried in 0·5 M-sorbitol in 2·5 % peptone were reconstituted with 0·25 ml. distilled water added as drops delivered at various rates. Experiments A and B were carried out with the same dried culture but stored for different periods. In expt. C, the counts are the means from duplicate samples. The common maximum rehydration rate of 20 mg. water/sec./mg. dry matter is the approximate value given by direct pipetting of 0·25 ml. water to a dried sample. Drop size: △, 1/35 ml.; ○, 1/270 ml.; ▲, 1/28 ml.; ●, 1/200 ml.

water flux will be a function of the solute concentration at a distance, and an inverse function of the rate at which the solute molecules themselves enter the cell. Such a view is in accord with the experimental facts summarized in Fig. 5, and with the different rates at which members of this series of solutes penetrate into bacteria (Mitchell & Moyle, 1956). Attempts to obtain more direct evidence for the influence of rehydration rates are described in the following section.

Rehydration at various rates. Some attempts were made to control the rate of rehydration of dried cultures of *Vibrio metchnikovi* with water added in the vapour phase but with the rates realized, this procedure was highly lethal. The maximum rehydration rate obtained was about 10^{-3} mg. water/sec./mg. dry matter. The extreme slowness of rehydration by this method probably accounted for its detrimental effect.

Considerably faster rates were obtained by adding water in the form of a mist from a glass atomizer. Rehydration of dried *Vibrio metchnikovi* suspensions with mists delivered at different rates suggested that slow rehydration may produce a recovery several times greater than that from direct rehydration with liquid water. There was, moreover, some indication of an optimum rehydration rate of the order of 10^{-2} mg. water/sec./mg. dry matter. These same trends were demonstrated more conclusively however in the following experiments in which water was added in the liquid phase as small drops.

By fitting capillary tubes of suitable bore to the delivery tip of a burette, rehydration could be carried out at varying rates with drops of a required size. When 0·2 ml. replicate samples of dried *Vibrio metchnikovi* suspension were reconstituted with 0·25 ml. distilled water added as drops in this manner, the recovery of viable organisms was markedly increased by slower rates of addition, for a wide range of drop-sizes (Fig. 6). The best recovery was produced by rehydration at 10^{-1} to 10^{-2} mg. water/sec./mg. dry matter. It was again evident, as in rehydration with water vapour and mists, that rates of less than 10^{-2} mg. water/sec./mg. dry matter were detrimental, although the relative damage at these rates was very variable. The viable counts obtained at the optimum rehydration rates were from about five to a hundred-fold greater than those given by rapid pipetting of 0·25 ml. water on to the dried suspensions.

Rehydration at various temperatures. The effect of the temperature of rehydration of *Vibrio metchnikovi* was examined in several fluids; Fig. 7 summarizes the results of an experiment in which rehydration was carried out at four temperatures in 2 ml. of each of four fluids. The actual rehydration operations at each temperature were carried out in a constant-temperature room, at the appropriate temperature, all equipment and solutions being at the operating temperature. After the initial rehydration the ampoules were transferred to room temperature before further dilutions in saline were made. Inspection of Fig. 7 shows that for each fluid, rehydration at 37° resulted in lower viable counts than at 0°, 11° or 22°. For the two most concentrated solutions, however, the adverse effect at 37° was decreased. Other experiments showed that rehydration in 0·2 ml. also decreased the deleterious effects of rehydration at 37°. There was no indication that rehydration at 0° led to any decrease in viability.

Other experiments with *Vibrio metchnikovi* showed that the lethal effects which followed the transfer of undried organisms to hypotonic solutions were also much greater at 37° than at the lower temperatures. It was not possible to relate these changes in viability to loss of substances which absorbed at wavelength 256 mμ, since in all cases the concentration of these substances in the cell-free supernatant fluids was much greater than with other bacteria studied. This large amount of leakage was doubtless a consequence of the rather large mortality which occurred with all drying and rehydration treatments.

Experiments with other organisms

The influence of rehydration on a wide range of organisms was examined mainly by reconstituting 0·2 ml. replicate samples of dried suspensions in different volumes of water. Viable counts of suspensions of *Pasteurella multocida*, *Salmonella oranienburg*, *S. newport*, *Escherichia coli*, *Pseudomonas fluorescens*, *Serratia marcescens*, *Bacillus subtilis*, *B. stearothermophilus* (spores), *Lactobacillus casei*, *Streptococcus lactis* and *Staphylococcus aureus* were unaffected by differences of 0·25 to 16 ml. in the volume of water used for reconstitution, even when prolonged storage had decreased the overall viability to less than one-thousandth of the original value. On the other hand,

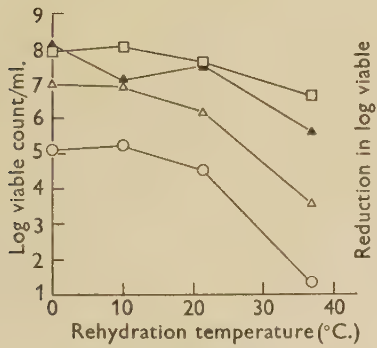

Fig. 7

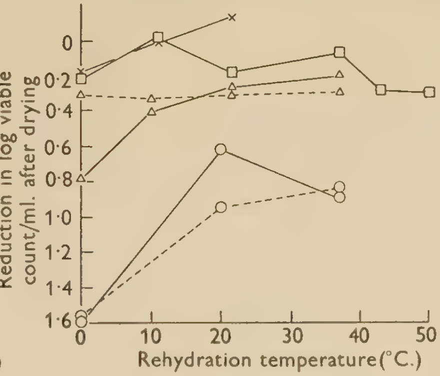

Fig. 8

Fig. 7. Survival of dried *Vibrio metchnikovi* as a function of the rehydration temperature. Viable counts from 0·2 ml. samples of a culture, dried in 0·1 M-sorbitol, and reconstituted at various temperatures with 2 ml. of: □, sucrose solution (0·980 a_w [1·03 molal]); ▲, NaCl solution (0·980 a_w [0·61 molal]); △, NaCl solution (0·995 a_w [0·15 molal]); ○, distilled water.

Fig. 8. Survival of dried suspensions of various organisms as a function of the rehydration temperature. Viable counts from 0·2 ml. samples of cultures, dried in 0·1M-sorbitol, and reconstituted at various temperatures with 2 ml. rehydrating fluid. ×, *Staphylococcus aureus*; □, *Saccharomyces cerevisiae*; △, *Serratia marcescens*; ○, *Escherichia coli*; strain B. The solid lines represent rehydration with distilled water, and the broken lines NaCl solution (0·960 a_w [1·2 molal] for *Serratia marcescens* and 0·980 a_w 0·61 molal] for *E. coli*).

suspensions of *Mycoplasma mycoides* dried in peptic digest + serum mixtures with added sucrose, gave viable counts which were up to 1000 times greater when the volume of water used was decreased from 2·0 to 0·25 ml. Similar comparisons for coliphages T2 and T5 dried from various media gave counts which were up to 100 times greater for the smaller volume, although with coliphage T2 the results varied somewhat in different suspending fluids.

In view of the results of Herrera *et al.* (1956) some tests were made with the rehydration of *Saccharomyces cerevisiae* and *Serratia marcescens* at different temperatures. The results, together with those for other experiments with *Escherichia coli* and *Staphylococcus aureus*, are summarized in Fig. 8, from which it may be seen that rehydration in cold water was disadvantageous for all three bacteria, but that no such effect was observed with the yeast. The damaging effect of rehydrating *S. marcescens* in water at 0° was cancelled by rehydration in NaCl solution; this was not so for *E. coli*. Of the three bacteria only *S. aureus* showed any consistent increase in leakage of 256 mμ-absorbing materials after rehydration in cold water, the losses at 0° being some 4–5 times greater than those observed at 22°. These results are, therefore, in the direction expected from the report of Sant & Peterson (1958) and contrast with the effect of temperature previously found with *Vibrio metchnikovi*. The different responses of the latter organism and of *S. marcescens* were confirmed in an experiment in which mixtures of the two organisms were dried together and replicate ampoules rehydrated at 0 and 37°. In comparison with the counts after rehydration at 0°, rehydration at 37° increased the population of *S. marcescens* by *c.* 30 % and decreased that of *V. metchnikovi* by *c.* 80–85 %.

DISCUSSION

It can be assumed that in restoring water to dried organisms there will be some finite rate of rehydration at which the damage to the cells will be least. Very slow rates will increase the damage due to storage in the presence of some water (Monk & McCaffrey, 1957; Scott, 1958); extremely fast rates will doubtless tend to disrupt cellular components as a result of differential expansion or swelling. A problem of rehydration is, therefore, to define the rate of rehydration most suitable for retaining the functional and structural integrity of the organism. The present paper provides some experimental evidence which suggests that the rate of rehydration is important, at least for *Vibrio metchnikovi*. The rates shown in Fig. 6 are, of course, mean values based on the discontinuous addition of water, and they should not be expected to apply when water is added continuously. It may be noted, however, that the trends were not greatly affected by a tenfold difference in drop size. For the conditions of these experiments the best survival resulted when the mean rates of addition of water were between 0·01 and 0·1 mg./sec./mg. dry matter, or between 1 and 10 % of the dry matter/sec. For a fully hydrated organism containing water equal to 300 % of the dry weight, the time required to restore this amount of water would then be from 30 to 300 sec. Although only very approximate, these estimates show the order of magnitude of the most favourable rates in comparison with the 'instantaneous' rates attainable with liquid water. The results with aqueous solutions (Figs. 3, 5) furnish only suggestive evidence that the rate of rehydration is important, and they cannot be used to estimate the most beneficial water flux. The experiments with aqueous solutions have shown, however, that for *Vibrio metchnikovi* exposure to dilute solutions is a major cause of death when relatively large volumes of water or dilute solutions are employed. The fact that dried organisms proved to be more susceptible to dilute solutions than undried organisms (Fig. 4) is of some interest, although there is no evidence that this is true for organisms other than *V. metchnikovi*. It is of interest that the most favourable a_w value for rehydrating *V. metchnikovi* was usually between 0·96 and 0·98, considerably less than the optimum for growth, which was shown by Marshall & Scott (1958) to be about 0·995 for this osmotically sensitive organism. Iljin (1953) discussed the need to control the rate of rehydration of dried plant cells, and stated that rehydration in sucrose solution was beneficial.

The methods used to assess the integrity of the cells will doubtless affect the probability of detecting any damage which the organisms may suffer during rehydration. For instance, Mitchell & Enright (1957) demonstrated that the fermenting and leavening ability of dried yeast was destroyed by certain rehydration procedures which did not impair viability; special rehydration procedures were necessary for retention of fermenting and leavening properties. Similarly, Sant & Peterson (1958) showed that rehydration treatments which caused only moderate leakage from yeast cells did not necessarily affect viability. The present results show that some of the bacteria whose viable counts were unaffected by rehydration in different volumes of water were, nevertheless, somewhat decreased in numbers by rehydration at low temperatures (Fig. 8). The greater tolerance exhibited by some organisms may simply indicate differences in degree rather than in the nature of their requirements. Perhaps conditions of rehydration which are essential for sensitive species will also decrease the chances of damage to more resistant organisms. On the other hand, evidence for differential responses to the temperature of rehydration (Figs. 7, 8) suggests that there may be real differences in the requirements of different organisms. Further work may show whether or not this is so, and may explain the possible interconnexion between solute requirements, temperature, pH value and the previous treatment of the organisms, as revealed by Wasserman & Hopkins (1957) and by some of the results presented above. From a practical point of view it is clear that successful rehydration of some micro-organisms will be aided by rehydrating with limited volumes of concentrated solutions or infusions and, where rehydration with liquid water is essential, by using no more water than is necessary, and controlling the rate of its addition.

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The Effect of Infection with Bacteriophage on the Electrokinetic Potential of *Rhizobium leguminosarum*

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SUMMARY: Infection with bacteriophage increased the electrophoretic mobility of pea nodule bacteria (*Rhizobium leguminosarum*) at pH 7, suggesting an alteration of the bacterial surface. This seemed to occur at about the middle of the latent period.

As the electrophoretic mobility of a bacterium in a constant environment depends on properties of the surface of the organism, alterations in mobility have been used as a means of detecting changes in the structure of the surface. Changes have been detected in this way during the germination of spores and during the initial stages of growth of the vegetative form (Douglas & Parker, 1958*b*), at the beginning of growth in a new medium, during transitions between 'smooth' and 'rough' forms (Moyer, 1936), and during treatment with enzymes (Dyar, 1948; Harris, 1953; Douglas & Parker, 1958*a*, *b*) or with bactericidal and related substances (Bradbury & Jordan, 1942; Cohen 1945). The purpose of this work was to find whether, and if so at what stage, infection with bacteriophage alters the bacterial surface in a way reflected in a change of electrophoretic mobility.

METHODS

A strain of pea nodule bacteria (*Rhizobium leguminosarum*) and a corresponding bacteriophage were used. The media and the method of poured plates for obtaining plaques were as previously described (Kleczkowska, 1945). Liquid cultures of the bacteria lysed by the phage, and passed through a Chamberland L3 filter, are referred to as 'stock cultures' of the phage; these were stored at 0°.

Electrophoretic mobility was measured in M/15 phosphate buffer (pH 7) at 0° in the 2 ml. Tiselius cell of a Perkin-Elmer Model 38 A electrophoresis apparatus under a gradient of 9·5 V/cm. As visual observation does not distinguish between phage-infected and non-infected bacteria in the same suspension, the optical system of the apparatus could not be used. Instead the mobilities were calculated from results of assays of fluids withdrawn separately from the ascending and descending limbs of the electrophoresis cell after periods of electrophoresis. The mobility of free bacteriophage, which was too dilute to give a visible boundary, was calculated similarly from results of assays. The descending limb and the bottom part of the electrophoresis cell were filled with the fluids to be examined, whereas the ascending limb contained only the buffer. The assays showed the proportions of phage or non-infected or infected bacteria which had left the descending limb and entered the ascending limb of the Tiselius cell when electrophoresis ended. Assuming that the tested materials were electrophoretically homogeneous, the distances they moved were computed by multiplying 5 cm. (the length of the limbs of the electrophoresis cell) by the proportions. The bacterial suspensions were stable enough for sedimentation under gravity not to affect them appreciably during the time of electrophoresis.

RESULTS

One volume of phage stock culture (containing about 10^9 phage particles/ml. by the plaque count) was added to nine volumes of a 24 hr. liquid bacterial culture (containing about 2×10^8 bacteria/ml. by the colony count) so that about half of the total number of bacteria became infected; the mixture was incubated at 25°. The latent period, i.e. the period between adding phage to the bacteria and the lysis of the first cells, lasts 90 min. and is followed by the first step of the logarithmic increase in the number of infective centres, which also lasts for 90 min. To measure the mobility of the bacteria at any desired stage of interaction with bacteriophage, the progress of the interaction was stopped by cooling to 0°. The bacteria were then washed in cold buffer to remove free bacteriophage, suspended in buffer and subjected to electrophoresis usually for about 30 min. The mobility of infected and of non-infected bacteria was then computed from the results of their assays (by the plaque count and by the colony count, respectively) in the fluids withdrawn from the descending and ascending limbs of the Tiselius cell. The mobility of non-infected bacteria was also measured, using bacterial suspensions to which no phage had been added.

Table 1 shows that in each experiment, when bacteria had been incubated for 60 or 120 min. at 25°, the phage-infected bacteria moved faster than non-infected bacteria (Expts. 6–10). When, however, incubation with phage lasted only 30 min., the mobilities of infected and non-infected bacteria did not differ within the same experiments (Expts. 4 and 5), and comparisons within experiments were much more consistent than comparisons between experiments. Only the difference between the means of mobilities of non-infected and of infected bacteria which had been incubated at 25° for 60 or 120 min., was therefore tested statistically. A statistical test seemed necessary because of the considerable variation between the results of different experiments. The probability of the two means (0·708 and 0·866) differing only by chance is, by the t test, one in more than a thousand, so that the difference can be taken as highly significant.

The electrophoretic mobility of phage-infected bacteria was obtained by measuring the mobility of infective centres; these would include not only infected bacteria, but also any free phage particles that might have been present in spite of the precautions to exclude them. The mobility of free phage was therefore measured and found to be *c.* $-0{\cdot}45 \pm 0{\cdot}02$ μ/sec./V/cm. It is obvious, therefore, that infection increased the mobility of the bacteria, for the infected bacteria moved faster than non-infected bacteria, whereas free phage moved more slowly than either. There seemed to be only one increase, which occurred between 30 and 60 min. of incubation at 25°, i.e. about the middle of the latent period. The timing must, however, be considered uncertain because of the considerable variation between the results of different experiments.

Table 1. *Electrophoretic mobilities of non-infected and of phage-infected* Rhizobium leguminosarum

Expts. 1–3 were made with bacterial suspensions to which no phage was added. Expts. 4–10 were made with mixtures of bacteria with phage incubated at 25° for indicated periods of time. The mobilities of non-infected bacteria in Expts. 6 and 9 were not estimated because of technical failures.

		Phage-infected bacteria Time of incubation at 25° after adding phage (min.)		
Expt.	Non-infected bacteria	30	60	120
	Mobility (μ/sec./V/cm.) towards anode			
1	0·65	—	—	—
2	0·66	—	—	—
3	0·72	—	—	—
4	0·69	0·69	—	—
5	0·72	0·75	—	—
6	—	—	0·84	—
7	0·75	—	0·88	—
8	0·70	—	0·93	—
9	—	—	—	0·77
10	0·77	—	—	0·91
Mean	0·708 ± 0·01463		0·866 ± 0·02837	

With the system used in this work nothing is known about the progress of phage-host interaction during the latent period. With some coliphages the middle of the latent period corresponds approximately with the end of the eclipse stage, when mature phage particles begin to appear within infected bacteria (Doermann, 1952; Anderson & Doermann, 1952). If it be assumed that the eclipse stage of the Rhizobium bacteriophage also ends at about the middle of the latent period, the increase in the electrokinetic potential of the bacterial surface would approximately coincide with the end of the eclipse stage.

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Cellulose Production by *Acetobacter acetigenum* and other *Acetobacter* spp.

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SUMMARY: The synthesis of cellulose by Acetobacter on media based on blackstrap molasses was studied. Methods are described for the determination of cellulose, and of growth as cell-N, in static culture. Different carbohydrate substrates were compared for cellulose production by *Acetobacter acetigenum* strain EA-I; hydrolysed molasses was found to give the largest yields. In media based on this substrate growth and cellulose production both reached their maximum values with a sugar concentration of 7·9 % (w/v) after 40 days' growth at 26–29°. The maximum conversion (18 %) of sugar to cellulose, however, was obtained with 1·5 % (w/v) sugar. Cellulose synthesis ceased when growth stopped, even when the sugar in the medium was not exhausted, indicating that cellulose was synthesized only by the actively growing organism. The cellulose produced/mg. cell-N and % cellulose in the pellicles decreased with increased sugar concentration in the medium. Other Acetobacter strains were examined in this medium; the cellulose yields varied over a wide range, equivalent to conversions of 1·9–23·5 %.

The synthesis of cellulose by *Acetobacter* species has been the subject of many investigations since the first report by Brown (1886), but apart from the detailed work of Tarr & Hibbert (1931) and Woeber (1954) and the more restricted studies of Kaushal & Walker (1951), Barclay (1951) and Skopek (1955), relatively little attention has been given to the influence of cultural conditions on cellulose yields, and nothing has been published concerning the relationship between cell growth and cellulose synthesis. The present study was undertaken to find the conditions which give maximum yields of cellulose, especially with cheap and readily available substrates, with a view to possible large-scale production of this polysaccharide. The present paper describes experiments to determine the influence of some nutritional factors on cellulose production by *A. acetigenum* in media based on blackstrap molasses. Cellulose yields from three strains of *A. acetigenum*, six strains of *A. xylinum*, two strains of *A. kutzingianum* and one strain of *A. pasteurianum* are reported. Studies of cellulose synthesis in cultures grown statically on defined media and under aerated and agitated conditions will be reported separately.

METHODS

Organisms. Acetobacter acetigenum, East African strain, originally sent by Professor T. K. Walker (Manchester University) to the Hankey Culture Collection (HCC) maintained at this Institute, was divided into two variants on the basis of cellulose yields. The culture, maintained for two years on the hydrolysed molasses medium described below, has been designated strain EA-I to distinguish it from the culture maintained on glucose + Marmite + peptone medium, designated strain EA-II; the same strain deposited by Professor Walker at the National Collection of Industrial Bacteria (NCIB) was obtained from the NCIB as strain NCIB 8132. *A. xylinum* strain HCC B-51 was obtained originally from the NCIB. Strains HCC B-150 and HCC B-151 were isolated locally by Miss M. B. Morris (HCC) from stout and from cane juice vinegar, respectively. Strain Hestrin was obtained from Dr S. Hestrin (Hebrew University, Jerusalem). Strain 472 was obtained from the Division of Applied Microbiology, National Research Council, Ottawa. *A. xylinum* strain Walker C (NCIB 8754), *A. kutzingianum* strains Walker A (NCIB 8752) and Walker D (NCIB 8755) and *A. pasteurianum* strain Walker B (NCIB 8753) were obtained from Professor T. K. Walker; it should be noted that these strains of *A. kutzingianum* and *A. pasteurianum* are atypical.

The organisms were maintained on the following medium (%, w/v): hydrolysed molasses (prepared as described below), 2–3 total sugar; $(NH_4)_2SO_4$, 0·3; KH_2PO_4, 0·5: Marmite, 0·5; adjusted to pH 6. The cultures were grown at tropical room temperature (26–29°) and subcultured at intervals of 4 weeks.

Media. Details of the various media used are given with the results. The blackstrap molasses contained 65% (w/w) total sugar (42% sucrose, 23% reducing sugar) and 0·37% (w/w) total-N. For hydrolysis the molasses was diluted fivefold with distilled water and treated with one-twentieth of its volume of a crude invertase solution prepared from fresh brewery yeast as described by Hestrin, Feingold & Schramm (1955). Hydrolysis was complete in 24–48 hr. at room temperature, after which bentonite (1%, w/v) was added and the solution clarified by boiling for 20 min. and filtering while hot.

Inoculation. Culture liquid from well-shaken test-tube cultures, activated by two or three previous transfers at 3-day intervals, was used when less than 10 days old. The yield of cellulose at completion of growth was not sensibly affected by the size of inoculum, over the range 0·2–4 ml./100 ml. of sterile medium.

Culture method. Weighed 250 ml. flasks containing 100 ml. medium were inoculated and allowed to remain undisturbed on the bench at room temperature (26–29°).

Analytical methods. The flasks were weighed before analysis and the decrease in weight, assumed to be loss by evaporation, was taken into account when calculating the residual-sugar results. The flasks were thoroughly shaken and the culture fluid decanted for potentiometric pH measurement and residual sugar determination. When molasses was used the reducing sugars (glucose + fructose) were determined by the method of Somogyi (1952); the total sugar was determined by the anthrone method of Fairbairn (1953); the sucrose present was calculated by difference. When glucose, sucrose, soluble starch or hydrolysed molasses were used, the residual sugar was determined by the anthrone method. The sugar utilized by the cultures was calculated by difference between the residual sugar found and that initially present in the medium.

The pellicles were left in the flasks and washed with repeated changes of distilled water. Mercuric chloride solution (1 ml., 10%, w/v) was added to the first few changes of water to prevent the growth of contaminants. Thin pellicles were washed for 1–2 days and thick pellicles for periods up to 10 days, all with several changes of water daily. The final washings were free from sugar and soluble nitrogenous constituents. At this stage it was assumed that the pellicles, referred to as 'crude pellicles', contained only cellulose + organisms.

It was found possible to obtain a measure of growth in the cultures by determining the nitrogen content of washed crude pellicles, on the assumption that all the organisms were trapped in the pellicles. The reproducibility of results from replicate cultures indicated that any error caused by the loss of organisms during washing was small. The use of water to wash the crude pellicles was shown not to affect the nitrogen content of the Acetobacter organisms. When samples of a suspension of *Acetobacter acetigenum* were compared after standing at room temperature for 8 days in daily changes of water and physiological saline, respectively, the difference found in the cell-N content was negligible (organisms washed in water: 12·5 % N; organisms washed in saline: 13·0 % N). Since the wet crude pellicles were highly hydrated (*c.* 99 % water; Schramm & Hestrin, 1954), the weight of the pellicles in grammes was equivalent to their volume in millilitres; it was thus relatively simple to determine the volume of each wet crude pellicle by weighing it. For nitrogen determinations the pellicles were weighed in the flasks and an equal volume of 2N-NaOH added. The flasks were stoppered and put on a rotary shaker overnight to ensure complete dissolution of cell material from the pellicle. Control experiments with thick (1 cm.) pellicles showed that shaking for 10 hr. was sufficient for the purpose. Nitrogen determinations were carried out on 1 ml. samples of the alkaline extracts; from these results the nitrogen content of the crude pellicles was calculated.

To determine cellulose the alkali-extracted pellicles were washed first with dilute acetic acid (*c.* 1 %, w/v) until they remained acid to added methyl red indicator for several hours, and then with 8 changes of distilled water (150 ml.), allowing several hours for each wash, to remove the acid. The cellulose pellicles were hung on tared glass rods, allowed to drain overnight and then dried to constant weight at 105°. The cellulose obtained in this manner generally contained 0·2–0·4 % N, equivalent to 1·2–2·5 % protein, and was thus more than 95 % pure.

Calculation of results. The efficiency of conversion into cellulose was calculated as direct percentages in terms of the total sugar provided in the medium ('conversion of total sugar') and of the sugar utilized by the culture ('conversion of utilized sugar'). The relationship between cellulose yields and amount of organism obtained in the cultures was expressed as the 'cellulose: cell-N ratio' (mg. cellulose produced/mg. cell-N), or as the 'cellulose content of crude pellicle' which was calculated from the fraction

$$\frac{\text{cellulose} \times 100}{\text{cellulose} + \text{dry wt. organism}}.$$

The ratio which is obtained directly from the two measurements is independent of any assumptions and is reliable under all conditions, while the second term depends on the accuracy with which the relationship between cell-N and dry wt. organism is known. The two terms are related by the expression:

$$\text{cellulose content of crude pellicle} = \frac{\dfrac{\text{cellulose}}{\text{cell-N}} \times 100}{\dfrac{\text{cellulose}}{\text{cell-N}} + \dfrac{100}{\%\text{N in cell}}}.$$

In Fig. 1 values for the ratio cellulose:cell-N, ranging from 0 to 30, are related to the cellulose content of crude pellicle calculated on the basis of 14·9 % N in the organisms.

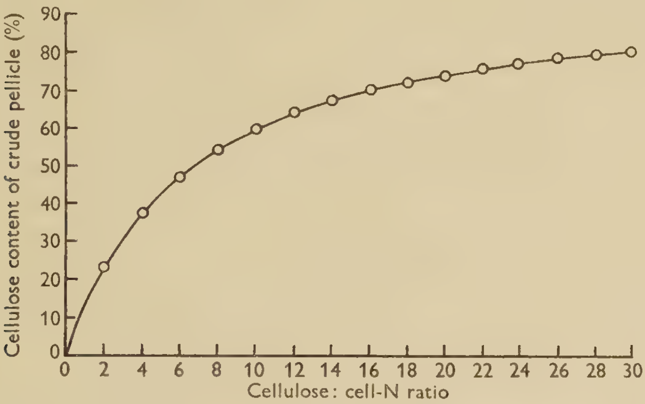

Fig. 1. Relationship between cellulose:cell-N ratio and cellulose content of crude pellicle in cultures of *Acetobacter acetigenum*, calculated for organisms containing 14·9 % N.

RESULTS

Cellulose production on dilute molasses media

Acetobacter acetigenum strain EA-I growing in dilute molasses medium produced the largest cellulose yields in a preliminary experiment in which the cellulose production of a number of strains was compared. This strain was therefore used in the main experiments. The behaviour of strain EA-I in dilute molasses medium is illustrated in Fig. 2. The rate of cellulose production was initially rapid; more than 75 % of the total yield was synthesized in 9 days. Subsequent synthesis was much slower; small increases in yield were detected up to 46 days. Although cellulose synthesis was slow after 9 days, sugar utilization continued at an undiminished rate until the 16th day, by which time most of the reducing sugar had been used. The organism was unable to use the sucrose in the molasses; the slight utilization in the later half of the culture period was probably aided by hydrolysis of some of the sucrose under the mildly acid conditions, as suggested by the slight increase in the residual reducing sugar found toward the end of the cultural period. The pH values of the cultures remained relatively stable throughout. The conversion of sugars into cellulose on this medium was of a very low order, being only 1·5 % of the total sugar and 7·3 % of the utilized sugar at the maximum. The nature of the inoculum made little difference to the results.

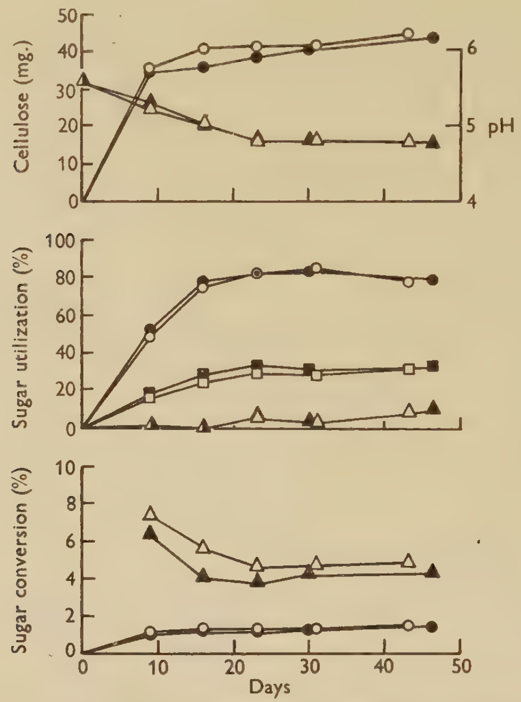

Fig. 2. Cellulose production, sugar utilization and conversion, and pH changes during growth of *Acetobacter acetigenum* strain EA-I in 100 ml. dilute molasses medium (3 %, w/v, total sugar; initial pH 5·6) inoculated with pellicles from 4-day test-tube cultures or with 4 ml. liquid from 24 hr. cultures. Results for cultures inoculated with pellicles shown as filled points (● ▲ ■) and with culture liquid as outline points (○ △ □). Top: cellulose yields (○ ●), pH (△ ▲). Middle: utilization of reducing sugar (○ ●), sucrose (△▲), total sugar (□ ■). Bottom: conversion of total sugar (○ ●), utilized sugar (△ ▲).

Inhibition of cellulose synthesis by salts

The inability of *Acetobacter acetigenum* strain EA-I to utilize sucrose (two-thirds of the total sugar in the molasses) indicated the importance of studying the behaviour of the organism in hydrolysed molasses. A preliminary experiment with molasses hydrolysed with hydrochloric acid and neutralized with sodium hydroxide showed that the resulting sodium chloride concentration (2·5 %, w/v) in the medium prevented growth. The sensitivity of this strain to sodium chloride and other salts was therefore examined.

Instead of hydrolysing molasses with various acids and neutralizing afterwards it was simpler to add known concentrations of salts directly to the medium. To make the conditions like those which would obtain in an acid hydrolysate of molasses it was desirable to add the salts to hydrolysed as well as unhydrolysed molasses. A sample of molasses was hydrolysed with crude yeast invertase to avoid the complications of acid hydrolysis. The effect of six salts (representing the products of hydrochloric, sulphuric, nitric, acetic and formic acid neutralized with sodium hydroxide, and of hydrochloric acid with potassium hydroxide) was examined over the range 0·05–2% (w/v) with molasses and hydrolysed molasses (both 2·4%, w/v, total sugar), and in the medium of Schramm & Hestrin (1954) with molasses, hydrolysed molasses and glucose (all 2·4%, w/v) as separate substrates.

Sodium acetate and nitrate showed stimulation, not inhibition, over the range of concentration examined; the other salts were found to be inhibitory at concentrations independent of the medium (sodium formate, 0·037 M; sodium sulphate, 0·036 M; potassium chloride, 0·137 M; sodium chloride, 0·171 M). With sodium chloride after 13 days of incubation thick pellicles were found in the media with 0·8%-NaCl (0·137 M), and very slight pellicles at 0·9% (0·154 M) and 1% (0·171 M). Inoculation into fresh salt-free medium from a 1% NaCl culture gave very retarded growth, but on further subculture into fresh medium the growth returned to its normal rate. It was decided to eliminate the complications of the salt effect in further experiments with molasses by using yeast invertase instead of acid for hydrolysis.

Comparison of cellulose yields from hydrolysed molasses, molasses, glucose, sucrose and starch

A comparison was made of cellulose production and sugar utilization by *Acetobacter acetigenum* strain EA-I with different substrates in the following media:

Medium A. Henneberg (1926) medium with the addition of Marmite as used by Professor M. Stacey (1955, personal communication); (%, w/v): carbohydrate, 2; $(NH_4)_2SO_4$, 0·3; KH_2PO_4, 0·3; $MgSO_4.7H_2O$, 0·2; Marmite, 0·5; adjusted to pH 5; ethanol (1%, v/v) was added after sterilization.

Medium B. (Professor M. Stacey, 1955, personal communication); (%, w/v): carbohydrate, 4; KH_2PO_4, 0·02; $MgSO_4.7H_2O$, 0·02; Marmite, 0·4; glacial acetic acid, 1% (v/v); no pH adjustments; ethanol (2%, v/v) was added after sterilization.

Medium C (Tarr & Hibbert, 1931); (%, w/v): carbohydrate, 2; asparagine, 0·1; KH_2PO_4, 0·5; NaCl, 0·1; adjusted to pH 5; ethanol (1%, v/v) was added after sterilization.

Medium D (Tarr & Hibbert, 1931); medium C + peptone, 0·1% (w/v); $MgSO_4.7H_2O$, 0·05% (w/v); $FeSO_4$, trace.

Medium E (Tarr & Hibbert, 1931); medium C + Marmite, 1% (w/v); $MgSO_4.7H_2O$, 0·05% (w/v); $FeSO_4$, trace.

Medium F (Schramm & Hestrin, 1954); (%, w/v): carbohydrate, 2; peptone, 0·5; Na_2HPO_4, 0·27; Marmite, 0·5; citric acid, 0·115; adjusted to pH 6.

Each medium was used with five different carbohydrate sources: hydrolysed molasses, molasses, glucose, sucrose and soluble starch. To ensure adaptation to each basal medium + carbohydrate mixture the organism was grown through four serial subcultures in each mixture before the appropriate flasks were inoculated. Growth on media with glucose, sucrose or soluble starch was slower, requiring 5–7 days between each subculture, than with molasses or hydrolysed molasses, which required 2–3 days. Growth was also relatively slow on basal medium D + glucose, sucrose or starch, showing the need for yeast extract or some growth factor for good growth. Growth was so slow on basal medium C + glucose, sucrose or starch and on medium F + sucrose or starch that they were discontinued. The flasks were each inoculated with 5 ml. adapted culture and incubated for 21 days. The pellicles differed considerably in appearance depending on the medium and added carbohydrate.

The results shown in Table 1 are the average values found for triplicate cultures. Pellicle thickness was found not to be always a reliable indication of cellulose yield. For example the pellicles from media B + glucose, B + sucrose, C + molasses, D + hydrolysed molasses, D + molasses and E + sucrose were all about 0·7 cm. thick but contained 97, 52, 137, 225, 177 and 42 mg. cellulose, respectively. These results suggest that considerable differences in density between pellicles may arise from growth on different media and carbohydrates.

The final pH values of the cultures depended on the nature of the carbohydrate source. All the cultures which contained glucose, sucrose or starch had pH values consistently lower than those containing molasses or hydrolysed molasses: these latter remained closer to the optimum pH range for cellulose synthesis.

The general degree of utilization of each carbohydrate source was independent of the basal medium, and, with three exceptions, reached the following values: hydrolysed molasses, 80–90 %; molasses, 42–65 %; glucose, 14–20 %; soluble starch, 12–15 %; sucrose, 5–10 %. The exceptional utilization results obtained with the cultures grown in medium B + molasses, medium B + hydrolysed molasses and medium F + glucose, are believed to have been due to the presence in these media of sparing adjuncts (ethanol + acetic acid in basal medium B; citrate in basal medium F). The behaviour of these adjuncts is discussed elsewhere (Dudman, 1959). The significance of the utilization figures for reducing sugar and sucrose in molasses media cannot be assessed because their relative final values may have been the direct result of utilization, or more reducing sugar may have been consumed and some replaced by sucrose hydrolysis. The apparent non-utilization of reducing sugar in basal medium B + molasses is believed to have arisen through the fortuitous hydrolysis of enough sucrose completely to replace the reducing sugar used.

Cellulose yields from each medium differed with the carbohydrate substrate. With the exception of medium F from which the highest yield was obtained with glucose, the yields from all the other basal media + carbohydrate sources were found to be in the following order: hydrolysed molasses > molasses > glucose > sucrose > soluble starch. The relatively low yields from molasses and hydrolysed molasses in basal medium F are believed to have been caused by the citrate present, which is also thought to be responsible for the high yield from glucose in this medium (Dudman, 1959).

Table 1. *Cellulose production by* Acetobacter acetigenum *strain* EA-I *grown on hydrolysed molasses (HM), molasses (M), glucose (G), sucrose (S) or soluble starch (SS) media for 21 days at 28–30° under static conditions*

Medium		Pellicle thickness (cm.)	Final pH value	Total carbohydrate in medium initially* (g.)	Carbohydrate utilization						Cellulose (mg.)	Carbohydrate conversion	
					Reducing		Sucrose		Total			Total (%)	Used (%)
					(g.)	(%)	(g.)	(%)	(g.)	(%)			
A	HM	1·2	5·75	2·22	—	—	—	—	1·79	80·6	268	12·1	15·0
	M	0·9	6·17	2·36	0·50	80·6	0·51	29·3	1·01	42·8	168	7·1	16·6
	G	1·1	3·41	2·32	—	—	—	—	0·45	19·4	79	3·4	17·6
	S	0·5	3·59	2·52	—	—	—	—	0·24	9·5	32	1·3	13·3
	SS	0·2	3·55	2·36	—	—	—	—	0·29	12·3	7	0·3	2·4
B	HM	1·2	5·20	4·10	—	—	—	—	1·02	24·9	311	7·6	30·5
	M	1·0	4·93	4·60	0	0	0·86	28·2	0·86	18·7	247	5·4	28·7
	G	0·7	4·07	4·25	—	—	—	—	0·84	19·8	97	2·3	11·5
	S	0·7	4·26	4·50	—	—	—	—	0·46	10·2	52	1·2	11·3
	SS	0·2	4·14	4·60	—	—	—	—	0·86	18·7	3	0·07	0·3
C	HM	0·9	5·75	2·20	—	—	—	—	1·99	90·4	217	9·9	10·9
	M	0·7	5·96	2·50	0·75	79·8	0·58	37·2	1·33	53·2	137	5·5	10·3
D	HM	0·6	6·43	2·29	—	—	—	—	1·99	86·9	225	9·8	11·3
	M	0·7	6·94	2·21	0·50	66·7	0·74	50·7	1·24	56·1	177	8·0	14·3
	G	0·2	3·19	2·13	—	—	—	—	0·31	14·6	130	0·6	4·2
	S	0·2	3·40	2·34	—	—	—	—	0·11	4·7	6	0·3	5·5
	SS	Very thin	5·07	2·10	—	—	—	—	0·25	11·9	—†	.	.
E	HM	0·9	5·74	2·15	—	—	—	—	1·83	85·1	268	12·5	14·7
	M	0·8	6·04	2·33	0·51	61·7	0·99	66·5	1·50	64·4	162	7·0	10·8
	G	1·4	3·50	1·99	—	—	—	—	0·38	19·1	101	5·1	26·6
	S	0·7	4·43	2·44	—	—	—	—	0·15	6·1	42	1·7	29·0
	SS	0·2	3·42	2·10	—	—	—	—	0·52	24·8	13	0·6	2·5
F	HM	0·3	6·89	2·13	—	—	—	—	1·85	86·9	126	5·9	6·8
	M	0·4	6·85	2·20	0·37	46·3	0·99	70·7	1·36	61·8	121	5·5	8·9
	G	0·5	4·63	2·14	—	—	—	—	1·91	89·2	177	8·3	9·3

* By analysis. † Disintegrated on purification.

Influence of hydrolysed molasses concentration on cellulose production

The effect of hydrolysed molasses concentration on growth and cellulose production by *Acetobacter acetigenum* strain EA-I was examined in a modified form of medium A (above). In addition to hydrolysed molasses the medium contained the following nutrients (%, w/v): $(NH_4)_2SO_4$, 0·3; KH_2PO_4, 0·5; Marmite, 0·5; adjusted to pH 6. The hydrolysed molasses used in this experiment was prepared by the action of crude yeast invertase on molasses with the least possible dilution; 1000 ml. molasses was diluted with 500 ml. water and hydrolysed with 200 ml. crude invertase, the action of which was complete in 48 hr. A series of dilutions of the hydrolysed molasses was prepared and sterilized separately from the other nutrients. After the sterile nutrient solutions were combined, the media were analysed for total sugar and the following concentrations found (%, w/v): 0·8, 1·5, 4·0, 7·9, 12·0, 15·4 and 23·2. Each flask was inoculated with 4 drops taken from a 4-day culture; after 9, 18, 27 and 40 days cultures at each concentration were analysed.

Although pellicle formation was not observed in the 15·4 % sugar cultures until after the 9th day, and in the 23·2 % sugar cultures until after the 18th day, sugar utilization (Fig. 3*a*, *b*) was found to have taken place in all the cultures by the 9th day. When expressed as % initial sugar provided in the medium (Fig. 3*a*) the rate of utilization was inversely related to the initial sugar concentration, but when expressed as the weight of sugar utilized (Fig. 3*b*) the rate and final weight of sugar utilized was directly related to the initial sugar concentration, except in the cultures at the two highest concentrations in which sugar was utilized abnormally slowly until the later stages of the culture period. Utilization was complete in 18 days in the 0·8 and 1·5 % sugar cultures, and in 27 days in the 4·0 % sugar cultures. By the 40th day the 7·9 % sugar cultures approached complete utilization. The cultures at the three highest sugar concentrations had not exhausted their sugar supply in 40 days and utilization was still continuing.

The pH values of the cultures at all stages of growth, except those at the two highest sugar concentrations, were related to the initial sugar concentration in the medium (Fig. 3*c*). The 0·8 % sugar cultures showed the smallest change from the initial pH value of the medium; the higher sugar concentrations gave progressively lower pH values.

Cellulose production (Fig. 3*d*) in some of these cultures was greater than in the previous experiments. The largest yield was 890 mg. in the 7·9 % sugar cultures after 40 days of incubation. Only those cultures which had exhausted their sugar supply reached maximum cellulose production in the 40-day growth period. The rates of cellulose production in the 1·5, 4·0 and 7·9 % sugar cultures were similar during the period of active synthesis, showing that sugar concentrations up to 7·9 % did not affect the rate of cellulose synthesis. The 12 % sugar cultures showed a slower rate of synthesis which remained constant between 9 and 40 days. The inhibitory effect of higher sugar concentrations was more clearly shown at the two highest concentrations; the 15·4 % and 23·3 % sugar cultures showed no evidence of cellulose production until after 9 and 18 days, respectively; after this, slight increases were found.

The highest values for the conversion of total sugar to cellulose (Fig. 3*e*) were obtained with the 1·5 % sugar cultures which had reached their final yield (17–18 %) by the 9th day. With the exception of the 0·8 % sugar cultures which reached a lower degree of conversion (13–14 %) than the 1·5 % sugar

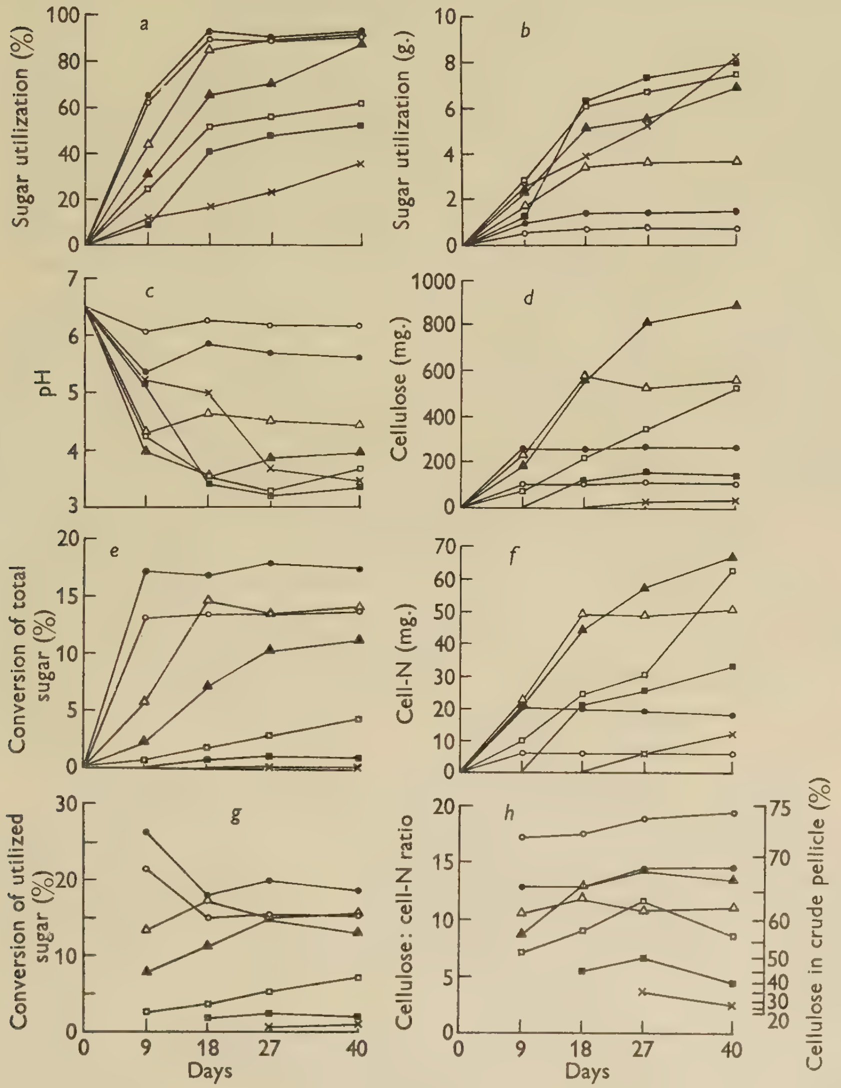

Fig. 3. The influence of total sugar concentration on static cultures of *Acetobacter acetigenum* strain EA-I grown in 100 ml. hydrolysed molasses medium: (*a*) sugar utilization (%); (*b*) sugar utilization (g.); (*c*) pH; (*d*) cellulose yield; (*e*) conversion of total sugar; (*f*) cell-N; (*g*) conversion of utilized sugar; (*h*) cellulose: cell-N ratio and cellulose content of crude pellicle. The sugar concentrations used were 0·8 % (○), 1·5 % (●), 4·0 % (△), 7·9 % (▲), 12·0 % (□), 15·4 % (■), and 23·2 % (×).

cultures all the other cultures showed conversions inversely related to the initial sugar concentration. The conversion of utilized sugar to cellulose (Fig. 3*g*) varied with the initial sugar concentration in the medium in the same manner as the conversion of total sugar, with the 1·5 % sugar cultures giving the highest values. The decrease in the conversion values found in the 0·8 and 1·5 % sugar cultures after 9 days was a consequence of the stoppage of cellulose synthesis in these cultures before all the sugar in the medium was exhausted; the subsequent sugar utilization was not accompanied by cellulose synthesis.

Growth (Fig. 3*f*) in all the cultures was similar in pattern to the cellulose production. During the period of active growth the 1·5, 4·0 and 7·9 % sugar cultures showed the same rate of growth, indicating the absence of inhibition by sugar concentrations up to 7·9 %. Growth in the cultures with the higher initial sugar concentrations was slower; the 12·0 % sugar cultures grew at about half the rate found in the more dilute cultures, while growth in the 15·4 and 23·2 % sugar cultures was slower and subject to the same long lag periods as cellulose synthesis.

The cellulose: cell-N ratios (Fig. 3*h*) showed a decrease with increased sugar concentration, indicating that less cellulose, relative to cell material, was synthesized as sugar concentration increased. To calculate the dry weight of organisms from the cell-N, the growth from two cultures grown in the 1·5 % sugar medium for 18 days was harvested following the procedure described by Hestrin & Schramm (1954). The organisms contained 14·9 % total-N and 2·1–2·5 % total carbohydrate (by micro-Kjeldahl and anthrone methods, respectively). The cell-N content was assumed to be constant at all sugar concentrations and the dry weight of organisms in all the cultures was calculated using this value of 14·9 %. The cellulose content of the pellicles varied widely, and decreased with increased sugar concentration in the medium from 71 to 74 % in cultures with 0·8 % sugar, to 28–35 % at the other extreme in the cultures containing 23·2 % sugar.

Comparison of growth and cellulose production by various Acetobacter strains grown in hydrolysed molasses medium

The growth and cellulose yields produced by the other Acetobacter strains capable of cellulose synthesis was examined on the following medium (%, w/v): hydrolysed molasses, 2·8 total sugar; $(NH_4)_2SO_4$, 0·3; KH_2PO_4, 0·5; Marmite, 0·5. The medium was adjusted to pH 6, and after sterilization ethanol (1 %, v/v) was added. The cultures were incubated for 30 days before analysis.

The results (Table 2) showed that the cellulose yields varied from 656 mg. (23·5 % conversion total sugar) with *Acetobacter xylinum* strain Hestrin, to 53 mg. (1·9 % conversion total sugar) produced by *A. acetigenum* strain EA-II, which may be considered to be a low-yielding variant of strain EA-I. *A. pasteurianum* and *A. kutzingianum* produced less cellulose than any of the strains of *A. xylinum* or than *A. acetigenum* strain NCIB 8132. The amount of growth calculated from the cell-N figures (assuming that all strains contained

Table 2. *Cellulose production by Acetobacter strains grown on hydrolysed molasses medium* (100 *ml.*) *for* 30 *days*

				Cell growth			Sugar conversion			
Acetobacter spp.	Strain*	Final pH	Sugar utilization (%)	N (mg.)	Weight† (mg.)	Cellulose (mg.)	Total (%)	Utilized (%)	Cellulose cell-N ratio	Cellulose in crude pellicle (%)
A. xylinum	Hestrin	4·80	94·1	23·5	158	656	23·5	25·0	27·9	81
A. xylinum	HCC B. 150	3·72	90·8	22·1	148	611	21·9	24·1	27·6	80
A. xylinum	472	4·21	85·7	33·6	226	490	17·6	20·5	14·6	68
A. acetigenum	NCIB 8132	4·97	76·1	23·4	157	367	13·2	17·3	15·7	70
A. xylinum	Walker C	4·11	89·2	27·1	182	327	11·7	13·1	12·1	64
A. xlyinum	HCC B. 51	4·05	84·9	20·4	137	308	11·0	13·0	15·1	69
A. xylinum	HCC B. 151	3·49	86·5	31·7	213	303	10·9	12·6	9·6	59
A. pasteurianum	Walker B	3·85	91·2	20·6	138	217	7·8	8·6	10·5	61
A. kutzingianum	Walker A	3·31	84·2	19·0	128	134	4·8	5·7	7·1	51
A. kutzingianum	Walker D	3·63	60·0	18·5	124	132	4·7	7·8	7·1	52
A. acetigenum	EA-II	4·70	96·0	24·5	165	53	1·9	2·0	2·2	24

* The strains are listed in the order of their cellulose yields.

† Calculated from cell-N results assuming cells to contain 14·9 % N.

14·9% N) varied between narrower limits (124–226 mg) than the cellulose yields, showing that the degree of growth was relatively uniform throughout the range of strains. There was no connexion between the dry weight of organism produced and the cellulose yields. *A. acetigenum* strain EA-II produced slightly more growth (165 mg.) than *A. xylinum* strain Hestrin (158 mg.), although the former strain produced 12 times less cellulose than the latter.

The cellulose:cell-N ratios (which varied between wide limits: 2·2–27·9) were related to the cellulose yields and showed erratically decreasing values with decreased cellulose production. The % cellulose in the crude pellicles decreased in the same manner, having its highest (81%) and lowest (24%) values with the strains which produced the largest and smallest yields of cellulose, respectively. The intermediate values were graded irregularly and followed the general trend.

DISCUSSION

The relationship between cellulose synthesis and the growth of Acetobacter organisms was examined for the first time in the present study. The results show that the rates of cellulose synthesis and growth were closely related and that cellulose was synthesized by *Acetobacter acetigenum* only during the period of active growth. This was clearly shown in the cultures grown in the media with 0·8 and 1·5% sugar, where the early stoppage of cellulose synthesis was linked with the stoppage of growth for which there was no obvious reason, since nitrogen and sugar were still available and the pH value was not unfavourable. The dependence of cellulose synthesis on growth in these cultures is surprising in view of the ease with which washed suspensions of Acetobacter synthesized cellulose from glucose under conditions which did not permit growth (e.g. Hestrin & Schramm, 1954). Sugar utilization however continued in these cultures at almost the same rate despite the stoppage of growth and cellulose synthesis, showing that sugar was being converted to products other than cellulose; their identification was not attempted. A similar stoppage of cellulose synthesis followed by undiminished sugar utilization was also observed when the organism was grown in diluted molasses medium.

The amount of cellulose synthesized/unit dry wt. organism was not constant under all cultural conditions. The cellulose: cell-N ratio decreased with increased concentration of sugar. The cellulose content of the crude pellicles, which is related to the cellulose:cell-N ratio, was thus also affected by the sugar concentration in the medium, and ranged from 71to 74% in the cultures grown in 0·8% sugar medium to 28–35% in cultures grown on 23·2% sugar. Brown (1886) found that the cellulose in the pellicles of *Acetobacter xylinum* varied from 35 to 62% depending on the growth conditions. *A. xylinum* strain Hestrin grown in hydrolysed molasses medium was found in the present study to produce pellicles containing 81% cellulose; Hestrin & Schramm (1954) found *c.* 38% cellulose in pellicles of the same strain grown on another medium.

The early observation by Brown (1886) that *Acetobacter xylinum* was unable to utilize sucrose was found to apply to *A. acetigenum* with regard to the sucrose in molasses. When grown in diluted molasses medium this organism used

80% of the available reducing sugar before attacking a small amount of sucrose. In the presence of other nutrients sucrose utilization reached higher values, but total sugar utilization in molasses media never reached the values obtained in hydrolysed molasses media.

Hydrolysed molasses, prepared by the action of yeast invertase, was the best sugar substrate, leading to the highest cellulose yields; molasses was the next best. Fructose, one of the best substrates for cellulose production (Tarr & Hibbert, 1931; Barclay, 1951; Skopek, 1955) and which accounts for *c.* 18% of the total sugar in molasses, and *c.* 50% of the total sugar in hydrolysed molasses, was probably the main factor responsible for the good cellulose yields from these substrates.

The observed sensitivity of *Acetobacter acetigenum* strain EA-I to inhibition of cellulose production by low concentrations of simple inorganic salts was in keeping with the finding of Tarr & Hibbert (1931) that cellulose yields from *A. xylinum* in defined medium were maximal when the sodium chloride concentration was 0·1% (w/v), and decreased to 56 and 31% of the maximum when the sodium chloride concentration was increased to 0·5 and 1·0% (w/v), respectively.

The results for the analysis of washed suspensions of *Acetobacter acetigenum* strain EA-I in which the nitrogen content was high (12·5–14·9%) and the total carbohydrate low (2·1–2·5%) were of the same order as the values obtained by Hestrin & Schramm (1954) for *A. xylinum* strain Hestrin, which contained 12% nitrogen and 0·5% 'apparent cellulose'.

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Cellulose production by *Acetobacter acetigenum* in defined medium

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SUMMARY: Growth and cellulose production by *Acetobacter acetigenum* strain EA-I was limited by nitrogen concentrations below 0·01 % (w/v) in glucose defined medium. Ammonium sulphate and asparagine + glutamic acid (50 % of each) were equivalent as nitrogen sources when compared on a nitrogen weight basis over the range 0·001–0·1 %-N; at higher concentrations ammonium sulphate decreased growth and cellulose synthesis, while asparagine + glutamic acid became stimulatory. When used as adjuncts in glucose defined medium, acetate, citrate and succinate at suitable concentrations increased growth and cellulose synthesis, by as much as 20–30-fold under the most favourable conditions. Ethanol stimulated growth but did not increase cellulose synthesis. The addition of calcium or magnesium carbonate to defined medium inhibited growth. The influence of glucose concentration in defined medium on growth and cellulose synthesis was relatively slight in the absence of succinate; in the presence of 0·084 M-succinate the cultures showed a much greater response to glucose concentration, with a maximum cellulose yield that was 7·6 times greater than the maximum in the absence of succinate, and was associated with a 1·9-fold increase in growth. In hydrolysed molasses medium the addition of succinate decreased growth and cellulose synthesis; slightly increased cellulose yields were obtained when ethanol and calcium carbonate were added.

In conjunction with the experiments previously described on cellulose production by *Acetobacter acetigenum* in complex media (Dudman, 1959), a study was made of growth and cellulose production in defined media. The use of defined media, free from complex nitrogenous compounds, was necessary to evaluate the influence of the nitrogen source, about which little is known except the early results of Tarr & Hibbert (1931).

The stimulatory effect of added ethanol on cellulose yields is well known. Ethanol cannot serve as the sole carbon source for growth and cellulose synthesis but when added to media containing suitable carbon substrates it increases the cellulose yields several fold (Tarr & Hibbert, 1931; Woeber, 1954; Minor, Greathouse, Shirk, Schwartz & Harris, 1954*a*, *b*). Acetate has been found to exert a similar stimulatory effect (Greathouse, Shirk & Minor, 1954). It is not known whether ethanol and acetate act by increasing cellulose synthesis specifically or whether growth is also increased. These adjuncts are believed to act by a sparing mechanism in which they are oxidized in place of part of the sugar substrate; this suggests that other compounds which are readily oxidized, such as the intermediate acids of the tricarboxylic acid cycle, might also act as stimulators of cellulose synthesis. In the present study the influence of ethanol, acetate, citrate and succinate was examined in defined medium. The most stimulatory of these (succinate) was also studied in hydrolysed molasses medium. The influence of glucose concentration on growth and cellulose synthesis in defined medium, in the absence and presence of succinate, was also investigated.

METHODS

Organism. The organism used, *Acetobacter acetigenum* strain EA-I, has been described (Dudman, 1959).

Media. Details of the various media are given with the results. Three growth factors, *p*-aminobenzoic acid, calcium pantothenate and nicotinic acid were added to all the defined media, in view of the requirement for growth factors of the B group found by Barclay (1951) and Woeber (1954). The factors were added to the media before sterilization in relatively high concentration (10 mg./l.) to allow for loss by heat destruction during sterilization. The trace element solution of Emery, McLeod & Robinson (1946), with the addition of cobalt nitrate (0·02 g./l.), was used to provide trace elements in the defined media.

Cultural conditions. The cultures were grown in 100 ml. medium in 250 ml. flasks, under static conditions at room temperature (26–29°).

Analytical methods. In addition to the methods described previously (Dudman 1959), residual nitrogen was determined by micro-Kjeldahl determinations on samples of culture liquid.

RESULTS

Influence of nitrogen concentration on growth and cellulose production

Two nitrogen sources, asparagine + glutamic acid (50 %, w/w, of each) and ammonium sulphate, were compared at the following nitrogen concentrations (%, w/v), 0·001, 0·0025, 0·005, 0·01, 0·025, 0·05, 0·1 and 0·2, in a basal medium containing (%, w/v): glucose, 2·0: KH_2PO_4, 0·5; NaCl, 0·1; $MgSO_4.7H_2O$, 0·1; *p*-aminobenzoic acid, 0·001; calcium pantothenate, 0·001; nicotinic acid, 0·001; trace element solution, 0·5 ml./l.; adjusted to pH 6. The media were sterilized by steaming for 20 min. on 3 successive days. The cultures were inoculated with 2 drops of a 1/10 dilution in physiological saline of liquid from a 2-day culture grown in hydrolysed molasses medium; this inoculum contained insufficient nitrogen (0·02 mg.) to affect the concentration in the medium. The cultures were incubated for 33 days.

Nitrogen concentrations below 0·01 % were limiting for glucose utilization, growth and cellulose synthesis (Fig. 1). The limiting concentration was the same for both the amino acid mixture and ammonium sulphate, showing that these nitrogen sources were equivalent in satisfying growth requirements when compared on an equal nitrogen weight basis. The glucose utilization level (Fig. 1*a*) remained constant at between 94–96 % for all the asparagine + glutamic acid concentrations above 0·01 %-N; there was no indication of inhibition at the highest concentration used. The cultures containing ammonium sulphate however, showed decreased glucose utilization at 0·2 %-N; at higher ammonium sulphate concentrations the organism failed to grow.

The final pH values (Fig. 1*b*) were of the same low order in all the nitrogen deficient cultures; in the ammonium sulphate cultures the final pH values remained low at all nitrogen concentrations. The asparagine + glutamic acid cultures were found to have higher pH values at the non-limiting nitrogen concentrations, reaching pH 6 at the three highest concentrations, presumably as a result of the increased buffering capacity of the media with high amino acid concentrations.

Cellulose yields (Fig. 1*c*) in the ammonium sulphate cultures at non-limiting nitrogen concentrations showed a slight increase from 30 mg. at 0·01 %-N to a maximum of 40 mg. at 0·1 %-N; the yield decreased to 26 mg. at 0·2 %-N. Cellulose yields in the asparagine + glutamic acid cultures remained constant (28–32 mg.) over the range 0·01–0·1 %-N, and showed a striking increase to 81 mg. at 0·2 %-N. The conversion of glucose to cellulose in all the cultures, however, was of a low order (Fig. 1*c*, *d*). Growth (Fig. 1*e*) was sharply limited at concentrations below 0·01 %-N. At higher nitrogen concentrations the cell-N values remained essentially constant, but showed relatively small changes reflecting the variation in cellulose yields. Assuming the cells grown under these conditions to contain 14·9 %-N (Dudman, 1959), the growth level attained (7·2–11·0 mg. cell-N/culture) was of the order of 48–74 mg. total dry weight of organism in the cultures not deficient in nitrogen.

The values of the cellulose: cell-N ratio (Fig. 1*f*) differed considerably between cultures deficient and adequate in nitrogen. The values were highest at 0·001 %-N and decreased rapidly with increased nitrogen in the medium to a value of 4·2 for both nitrogen sources at 0·01 %-N. At higher nitrogen concentrations the changes in the value of the ratio were relatively small. The cellulose content of the crude pellicles, calculated on the basis of 14·9 %-N in the cell dry matter, varied from about 75 % in the cultures with 0·001 %-N to about 35 % over the range 0·01–0·1 %-N; at 0·2 %-N the cellulose content was 52 and 30 % in the asparagine + glutamic acid and ammonium sulphate cultures, respectively.

Nitrogen utilization (Fig. 1*g*) showed a significant difference between the asparagine + glutamic acid and ammonium sulphate cultures. In the ammonium sulphate cultures with excess nitrogen, utilization remained constant (9·5–10·8 mg.-N/culture) showing that excess nitrogen was not used though available. In the asparagine + glutamic acid cultures excess nitrogen was used; at nitrogen concentrations above 0·01 %, utilization increased continuously, reaching 34 mg./culture at 0·2 %-N. Cell-N, as found, did not account for all the nitrogen utilized (Fig. 1*h*). Results for nitrogen recovery in the cultures with less than 0·01 %-N are omitted because of the relatively large experimental error involved in determining these small amounts. The recovery of utilized nitrogen in the ammonium sulphate cultures varied from 75 to 100 % over the range 0·01–0·2 % N; it is likely that the deviations from 100 % recovery indicate the error inherent in the method used for determination of the dry weight of organisms in these cellulose cultures; the method is based on the assumption that all the organisms are trapped in the pellicle. It is to be noted, however, that the recovery increased with increased nitrogen concentration.

In the asparagine + glutamic acid cultures the recovery values decreased progressively from 100% at 0·01%-N to 33% at 0·2%-N, which may be interpreted to mean that a mechanism operated in these cultures for the removal of nitrogen from the medium, in addition to removal by growth.

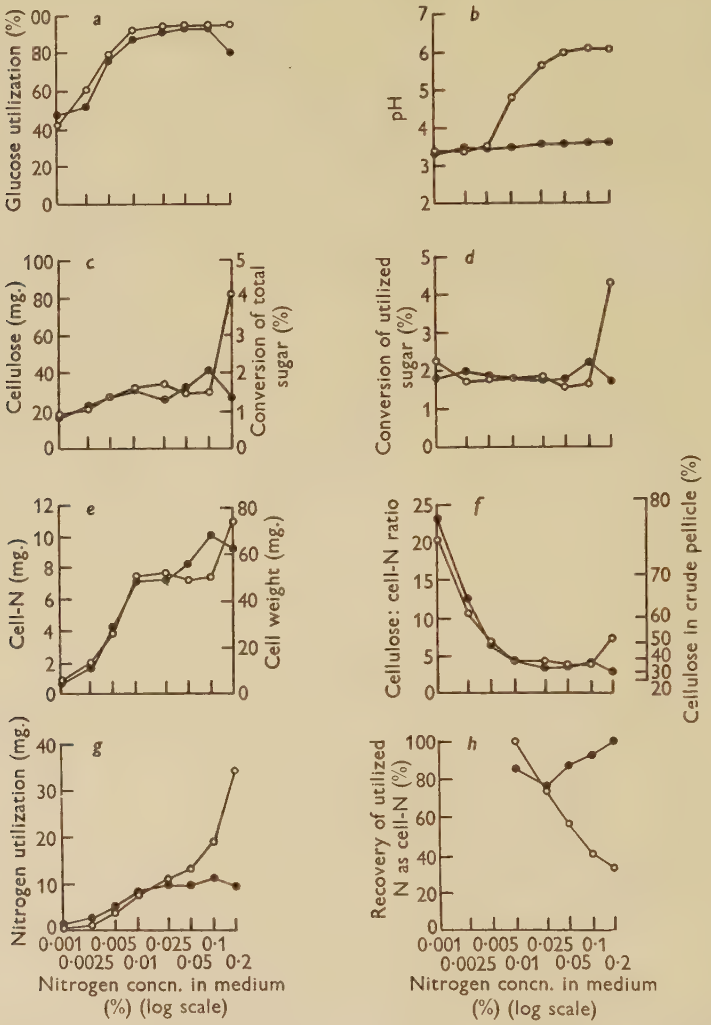

Fig. 1. Influence of nitrogen concentration in glucose defined medium on 33-day static cultures of *Acetobacter acetigenum* strain EA-I: (*a*) glucose utilization; (*b*) final pH value; (*c*) cellulose yield and conversion of total sugar to cellulose; (*d*) conversion of utilized sugar to cellulose; (*e*) cell-N and dry wt. of organism calculated assuming 14·9% N in organisms; (*f*) cellulose: cell-N ratio and cellulose content of crude pellicle; (*g*) nitrogen utilization; (*h*) recovery of utilized-N as cell-N. The nitrogen sources used were asparagine + glutamic acid (50%, w/w of each) (○) and ammonium sulphate (●).

Influence of adjuncts on growth and cellulose production in defined medium

The influence of ethanol, acetate, citrate and succinate on growth and cellulose production was examined at five concentrations (Table 1), equivalent to 0·1, 0·2, 0·4, 0·8, and 1·6 % (w/v) adjunct carbon, in an otherwise complete medium containing excess nitrogen. The defined medium above, containing 1·95 % (w/v) glucose and 0·47 % (w/v) ammonium sulphate (i.e. 0·1 %, w/v, N), and adjusted to pH 5·8, was used. The acetate, citrate and succinate adjuncts, prepared as solutions of the acids and adjusted to pH 5·8 with sodium hydroxide, were added to the medium before sterilization. Ethanol was added to sterile medium. Controls were set up for each adjunct, with 0·4 % (w/v) adjunct carbon in the basal medium without glucose.

Cultures were inoculated with a drop of 2-day culture in hydrolysed molasses medium, and incubated for 28 days. Growth occurred over the whole range of ethanol concentrations used, but the acid adjuncts were found to prevent growth at the following concentrations: citrate, 0·056 M; succinate, 0·333 M; acetate, 0·667 M. Growth was not observed in any medium containing adjuncts without glucose.

Table 1. *Adjunct concentrations required to give* 0·1, 0·2, 0·4, 0·8 *and* 1·6 % *(w/v) adjunct carbon in medium*

Adjunct carbon in medium (%)	Concentration			
	Acetic acid (M)	Citric acid (M)	Succinic acid (M)	Ethanol (M)
0·1	0·042	0·014	0·021	0·042
0·2	0·084	0·028	0·042	0·084
0·4	0·167	0·056	0·084	0·167
0·8	0·333	0·111	0·167	0·333
1·6	0·667	0·222	0·333	0·667

All the adjuncts had a marked stimulatory effect on glucose utilization (Fig. 2*a*). The final pH values (Fig. 2*b*) found in the cultures were very much dependent on the nature of the adjunct. The cultures with ethanol had the same low final pH values as the cultures grown in the basal medium, while the cultures with acetate, citrate and succinate, all of which are good buffers, were found to have higher pH values. Cellulose yields (Fig. 2*c*) showed a sharp differentiation between ethanol and the acid adjuncts. Cellulose synthesis was not stimulated by ethanol at any concentration, while very large increases were obtained with acetate, citrate and succinate. Cellulose yields from cultures with these three adjuncts were increased linearly by 0·1 and 0·2 % (w/v) adjunct carbon; at higher concentrations citrate inhibited growth and acetate gave smaller yields. The maximum yields were obtained with 0·4 and 0·8 % (w/v) succinate.

The influence of the adjuncts on growth (Fig. 2*f*) was not as distinct as in the case of cellulose production. Ethanol stimulated growth to higher levels than in the basal medium. The three acid adjuncts, at 0·1 % (w/v) carbon concentration, increased growth to the same level, but at higher concentrations the influence of the adjuncts varied. The largest increase in growth was obtained with succinate. The values found for the cellulose: cell-N ratio (Fig. 2*g*) in the adjunct cultures were all smaller at 0·1 % (w/v) adjunct carbon concentration than found in the cultures grown in the basal medium, showing that growth was more stimulated relatively, by the adjuncts at this concentration, than cellulose synthesis. Higher ethanol concentrations in the medium did not affect the value of the ratio. Higher concentrations of the adjuncts however gave increased values of the ratio.

These results suggested that succinate was the best adjunct for use in defined medium to obtain maximum growth and cellulose production.

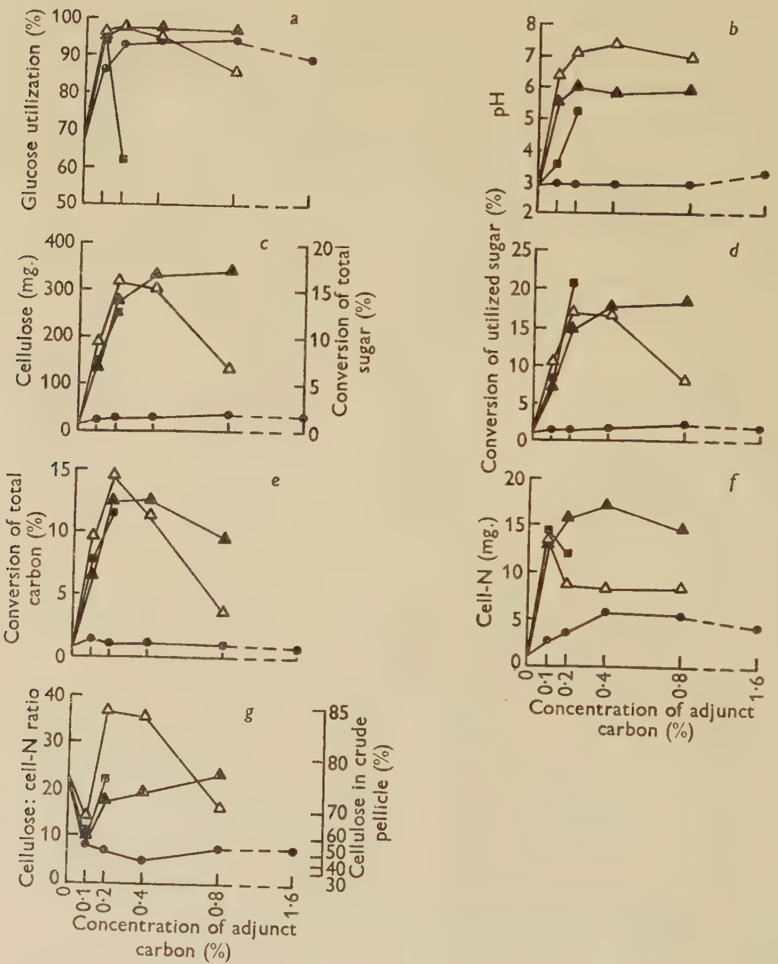

Fig. 2. Influence of acetate (△), succinate (▲), citrate (■) and ethanol (●) adjuncts in glucose defined medium on 28-day static cultures of *Acetobacter acetigenum* strain EA-I: (*a*) glucose utilization; (*b*) pH value; (*c*) cellulose yield, conversion of total sugar; (*d*) conversion of utilized sugar; (*e*) conversion of total carbon (i.e. glucose + adjunct carbon); (*f*) cell-N; (*g*) cellulose: cell-N ratio, cellulose content of crude pellicle (assuming 14·9 % N in organisms).

Attempts were made to examine the effect of ethanol at a more favourable pH by adding calcium carbonate (1–5 g./100 ml.) to the medium as a neutralizing agent, but without success. The use of calcium carbonate in the form of precipitated chalk, crushed marble or limestone, inhibited growth in defined medium even when the material was separately sterilized and added to cold sterile medium. The inhibitory effect was not overcome when additional amounts of the trace element solution and the growth factors were added immediately before inoculation with 1 ml. active 2-day culture. The same negative result was obtained with magnesium carbonate. The cause of inhibition was not investigated further.

Influence of adjuncts on growth and cellulose production in hydrolysed molasses medium

The possibility of increasing cellulose yields from hydrolysed molasses, by the use of suitable adjuncts, was examined in a medium containing (%, w/v) hydrolysed molasses, 2·0 total sugar; $(NH_4)_2SO_4$, 0·3; KH_2PO_4, 0·3; Marmite 0·5; adjusted to pH 6. The adjuncts used were: succinate (at the five concentrations shown in Table 1); succinate (0·084 M) + calcium carbonate (5 g./100 ml.); ethanol (0·167 M); ethanol (0·167 M) + calcium carbonate (5 g./100 ml.); calcium carbonate (5 g./100 ml.). The ethanol and sterile calcium carbonate were added to sterile medium. The cultures were inoculated with 2 drops of 3-day culture, and incubated for 49 days.

The addition of succinate to hydrolysed molasses medium had an adverse effect on growth and cellulose production, although growth was not completely inhibited by 0·333 M-succinate as in defined medium. Growth and cellulose yields were progressively reduced by increased succinate concentrations; the cellulose:cell-N ratio was constant however. The conversion of total sugar to cellulose decreased progressively from 28 % in the control medium to a value of 15 % at 0·333 M-succinate. Sugar utilization was not affected by the succinate adjunct. Of the other adjuncts tested, only ethanol and calcium carbonate, used separately, increased cellulose yields (by 6 and 10 %, respectively).

Influence of glucose concentration on growth and cellulose production in defined medium

The influence of glucose concentration in defined medium on growth and cellulose production was examined in the absence and presence of succinate. The basal medium contained (%, w/v): $(NH_4)_2SO_4$, 0·472 (i.e. 0·1 N); KH_2PO_4, 0·5; NaCl 0·1; $MgSO_4.7H_2O$, 0·1; *p*-aminobenzoic acid, 0·001; calcium pantothenate, 0·001; nicotinic acid 0·001; trace element solution, 0·5 ml./l.; adjusted to pH 6. Glucose was separately sterilized and added to the basal medium to give the following concentrations (%, w/v): 0, 1, 2, 5, 10, 15 and 20. Succinate was used at a concentration of 1·165 % (w/v), equivalent to 0·4 % (w/v) succinate-carbon. The cultures were inoculated with 2 drops of 2-day culture and incubated for 40 days. The results are shown in Fig. 3; results for cultures grown for 40 days in hydrolysed molasses medium at various sugar concentrations obtained in a previous experiment (Dudman, 1959) are included for comparison.

Glucose utilization (Fig. 3*a*, *b*) was complete in the 1 and 2% glucose cultures, with and without succinate. At higher concentrations the % utilization decreased progressively with increased glucose concentrations but was always greater in the cultures containing succinate. The final pH values (Fig. 3*c*) were higher in the succinate cultures.

Cellulose production (Fig. 3*d*) was greatly increased at all glucose concentrations when succinate was present in the medium. In the absence of succinate

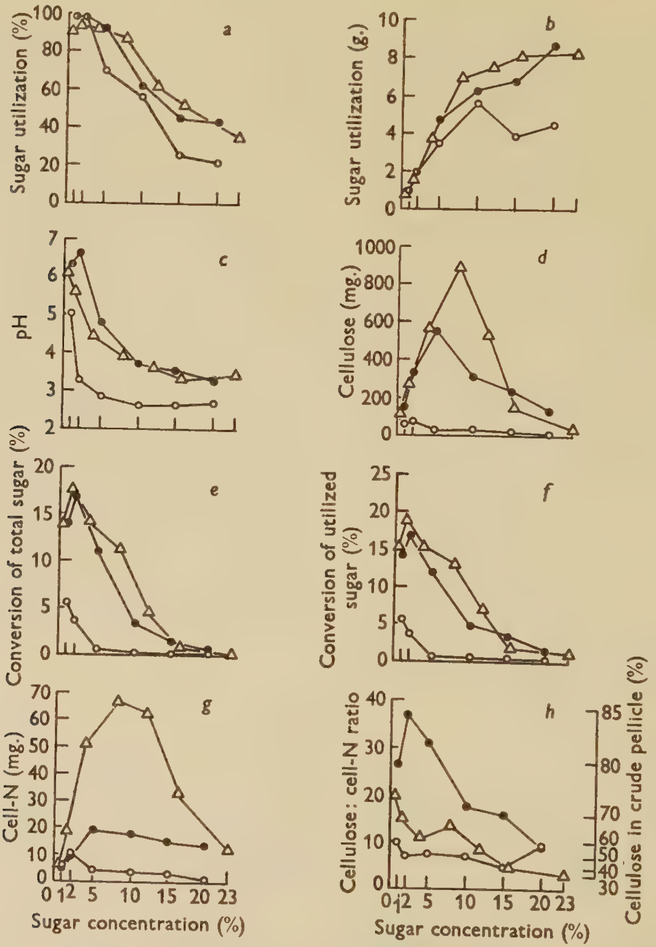

Fig. 3. Influence of sugar concentration in glucose defined medium on 40-day static cultures of *Acetobacter acetigenum* strain EA-I: (*a*) sugar utilization (%); (*b*) sugar utilization (g.); (*c*) final pH value; (*d*) cellulose; (*e*) conversion of total sugar; (*f*) conversion of utilized sugar; (*g*) cell-N; (*h*) cellulose:cell-N ratio; cellulose content of crude pellicle (assuming 14·9% N in organisms). Results are shown for cultures grown in glucose medium with (●) and without (○) succinate adjunct (0·084M), and in hydrolysed molasses medium without adjuncts (△).

the yields were low; the maximum was 73 mg. at 2% (w/v) glucose concentration. In the succinate cultures the maximum yield was 553 mg. at 5% (w/v) glucose concentration; yields decreased sharply at lower and higher glucose concentrations. Cellulose yields in the succinate cultures with 1–5% glucose closely paralleled those obtained when the organism was grown in hydrolysed molasses medium at equal sugar concentrations.

The influence of glucose concentration on growth (Fig. 3*g*) was also affected by succinate. In the medium without succinate, cell-N reached its maximal value (10·4 mg. N) at 2% glucose. In the succinate medium, growth at the 1 and 2% glucose concentrations was approximately the same as in the cultures without succinate, but increased to its maximal value (19·6 mg. N) at 5% glucose. Although the other results for the succinate cultures were similar to those obtained with cultures grown in hydrolysed molasses medium, there was a marked difference in the growth obtained in these two media; growth in the succinate cultures was much less than that obtained in hydrolysed molasses medium except at the lowest and highest sugar concentrations.

The values found for the cellulose:cell-N ratio in the succinate-free cultures ranged from 4·1 to 9·8, equivalent to 38–60% cellulose in the crude pellicles. In the cultures with succinate however the values found for the ratio were very much higher, with a maximum (36·5) at 2% glucose; the cellulose content of the crude pellicles grown in succinate medium ranged from 58 to 85%. The values of the cellulose:cell-N ratio were very much higher in the succinate cultures than in the cultures grown in hydrolysed molasses medium.

DISCUSSION

The synthesis of cellulose by *Acetobacter acetigenum* strain EA-I unlike the synthesis of extracellular polysaccharides by Aerobacter and Escherichia strains (Duguid & Wilkinson, 1953), was not stimulated when the organism was grown in media containing limited amounts of nitrogen source in the presence of excess glucose. Growth, cellulose yields and glucose utilization were all decreased when *A. acetigenum* was grown under these conditions.

Ammonium sulphate and asparagine + glutamic acid were equivalent on a nitrogen weight basis as nitrogen sources for the growth of *Acetobacter acetigenum*. Similar results were obtained with cultures grown on either nitrogen source over the range 0·001–0·1% (w/v) N, but a difference was found in the effect of these nitrogen sources at higher concentrations. Ammonium sulphate at concentrations equivalent to 0·2% N caused decreased growth and cellulose yields, while the equivalent concentration of asparagine + glutamic acid gave rise to increased growth and cellulose yield. There is no direct evidence to suggest the mechanism by which the amino acid mixture became stimulatory, but it is possible that it may have been the result of deamination, by the organism, of some of the asparagine and glutamic acid to oxalacetic and α-ketoglutaric acids, respectively, which being intermediates in the tricarboxylic acid cycle, would probably act in the same way as acetic, citric and succinic acids. Indirect evidence in support of this was the decreased recovery in the amino acid cultures, of utilized nitrogen as cell-N, as the nitrogen concentration of the medium increased.

Acetate, citrate and succinate were highly effective in stimulating cellulose synthesis by *Acetobacter acetigenum* in defined medium. The equal yields obtained from the three adjuncts, when used at concentrations equivalent to 0·1–0·2 % (w/v) adjunct carbon, suggests that they act in stimulating cellulose synthesis by the same mechanism, presumably a preferential oxidation of the adjunct to spare the glucose substrate for cellulose synthesis. This is supported by the recent observation of Schramm, Gromet & Hestrin (1957) that acetate, citrate, succinate and other intermediates of the tricarboxylic acid cycle are all readily oxidized to CO_2 by washed suspensions of *A. xylinum*. The buffering action of these adjuncts must also be an important additional factor in producing high cellulose yields by maintaining the cultural pH value within the optimum range for cellulose synthesis. The failure to obtain increased cellulose yields with ethanol added to defined medium may be explained by the low cultural pH values. The values found for the cellulose: cell-N ratio in the cultures with the acid adjuncts showed that the cellulose yield/unit weight of growth varied with the nature of the adjunct and its concentration.

The influence of glucose concentration on cellulose yields in defined medium was relatively slight in the absence of succinate. The addition of 0·4 % (w/v) succinate-carbon to the medium caused the cellulose yields to show a marked response to glucose concentration which paralleled that in hydrolysed molasses medium at sugar concentrations up to 5 %. The increased cellulose yields were associated with much smaller increases in growth, showing that under these conditions the succinate adjunct increased cellulose yields by specifically stimulating cellulose synthesis rather than by increasing growth generally. It had been hoped that cellulose yields from hydrolysed molasses medium, which were already large in the absence of adjuncts, would be increased further by the addition of succinate, but this was found instead to lead to diminished yields. No explanation can be given for the reversal in this medium of the stimulatory action of succinate.

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Acetate and Fumarate Permeases of *Mycobacterium smegmatis*

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SUMMARY: *Mycobacterium smegmatis* was grown on Lemco agar in the presence and absence of acetate and fumarate. Fumarate was oxidized rapidly by fumarate-grown organisms. Lemco-grown and acetate-grown organisms oxidized fumarate at an increasing rate after a lag period and this adaptation was inhibited by chloramphenicol. Disrupted bacterial preparations oxidized fumarate rapidly, irrespective of the growth medium. Acetate-grown organisms oxidized acetate at more than twice the rate of fumarate-grown and Lemco-grown organisms. There was slight adaptation to acetate oxidation by acetate-grown organisms but greater adaptation by Lemco-grown and fumarate-grown organisms. Acetate was oxidized by disrupted bacterial preparations. The evidence for the existence of acetate and fumarate permeases is discussed.

Recent studies have suggested that organic molecules may enter the bacterial cell by the action of specific transport mechanisms. Cohen & Monod (1957) reviewed the evidence for the existence of stereo-specific permeases in *Escherichia coli* and other organisms. Shilo & Stanier (1957) found that there were adaptive specific transport mechanisms for the three tartrate stereoisomers oxidized by pseudomonads. Clarke & Meadow (1959) suggested that there are adaptive permeases for the acids of the tricarboxylic acid cycle in *Pseudomonas aeruginosa*. Millman & Youmans (1955) found that all the necessary enzymes of the tricarboxylic acid cycle could be demonstrated in cell-free extracts of *Mycobacterium tuberculosis*, although few of the substrates could be oxidized by whole organisms. The impermeability of mycobacteria to many compounds which are normal cell metabolites, has long been thought to be related to the high lipid content of the cell wall. This investigation was designed to test the hypothesis that the impermeability of mycobacteria might be due to the absence of specific permeases, and to this end the oxidation of acetate and fumarate by *M. smegmatis* was studied.

METHODS

Mycobacterium smegmatis NCTC 8159 was obtained from the National Collection of Type Cultures. It was maintained by weekly subculture on slopes of Lemco agar (10 g. peptone; 10 g. Lab. Lemco; 5 g. NaCl; 10 g. agar; water to 1 l.; pH 7·6). Cultures incubated for 24 hr. in Lemco broth were used to inoculate Roux bottles each containing 100 ml. Lemco agar. Good growth was obtained after 2 days at 37°, but longer incubation produced more mucoid cells which were difficult to handle. After incubation, the bacteria

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were washed off the surface of the agar with about 100 ml. distilled water, centrifuged at 10,000 g for 5 min. and washed once with the same volume of distilled water. The organisms from four Roux bottles were resuspended in 0·05 M Sørenson's phosphate buffer (pH 7·6) and stirred mechanically to give a uniform suspension of 25–30 ml. for each series of experiments. Batches of organisms were also prepared which had been grown on Lemco agar to which either sodium acetate or sodium fumarate had been added to a final concentration of 0·4 % (w/v).

Suspensions were calibrated by measuring the optical density of suitable dilutions in an EEL (Evans Electroselenium Ltd.) colorimeter, and referring to a standard curve relating optical density to dry weight. Bacteria were disrupted by forcing the stock suspension through a small aperture at 4,000 lb./sq.in. in a bacterial press modified from an apparatus described by Milner, Lawrence & French (1950).

Manometric studies were carried out by the conventional Warburg technique. Flasks contained the equivalent of 2–4 mg. dry wt. of bacteria, or of 6–16 mg. dry wt. disrupted bacteria in 1 ml. 0·05 M-phosphate buffer pH 7·6. Substrate solution (0·4 ml. 4 %, w/v, Na salt) was added from the sidearm, and where required, 0·2 ml. chloramphenicol solution (1 mg./ml.) was added to the main flask and the volume made up to 3 ml. with the phosphate buffer; 0·2 ml. KOH (30 %, w/v) was added to the centre well. Experiments were carried out in duplicate and the figures given are means.

RESULTS

It was found that the rate of oxidation of acetate and fumarate by washed suspensions of *Mycobacterium smegmatis* depended on whether or not these compounds had been added to the growth medium. Organisms grown on Lemco agar showed adaptation to both acetate and fumarate oxidation. The initial rate of oxidation of acetate, and the linear rate in the presence of chloramphenicol were fairly high, but for fumarate the initial rate of oxidation and the rate in the presence of chloramphenicol were no greater than the endogenous rates. When the organisms were grown on acetate, the rate of acetate oxidation in the presence of chloramphenicol was more than twice that of the Lemco-grown organisms. In the absence of chloramphenicol, the rate of oxidation increased during the experimental period, but this increase was much less than with the Lemco-grown organisms. The acetate-grown organisms oxidized fumarate at a significant rate even in the presence of chloramphenicol, and the adaptation to fumarate oxidation in the absence of chloramphenicol, was much more rapid.

Organisms grown on fumarate oxidized fumarate rapidly at a constant rate from zero time, and the rate was not altered by the addition of chloramphenicol. Presumably therefore no adaptation to substrate was occurring. The fumarate-grown organisms oxidized acetate less rapidly than the acetate-grown organisms and there was some adaptation to acetate oxidation. The results for a typical series of experiments with whole organisms are shown in Fig. 1(*a*–*c*). There was some variation in activity with different batches of bacteria but this pattern of adaptive behaviour was consistent, except that after longer growth (3–4 days) on acetate agar, some batches of bacteria were found to oxidize acetate at a linear rate both in the presence and absence of chloramphenicol.

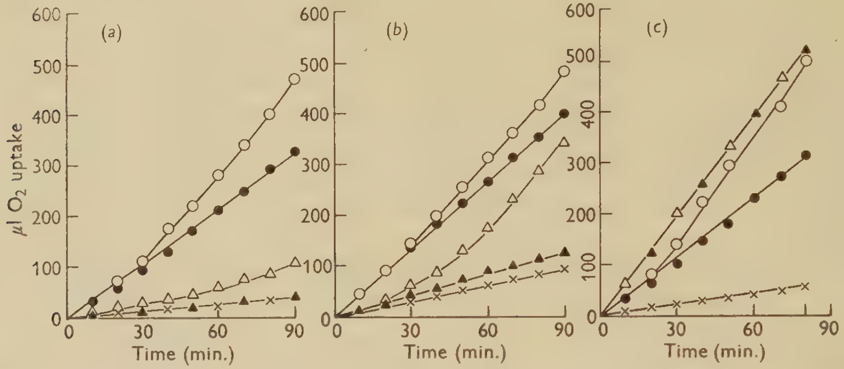

Fig. 1. Oxygen uptake by washed suspensions of *Mycobacterium smegmatis*; (*a*) Lemco-grown (1·2 mg. dry wt./ml.); (*b*) acetate-grown (1·3 mg. dry wt./ml.); (*c*) fumarate grown (0·8 mg. dry wt./ml.). ×—×, no substrate; ○—○, acetate; ●—●, acetate + chloramphenicol; △—△, fumarate; ▲—▲, fumarate + chloramphenicol.

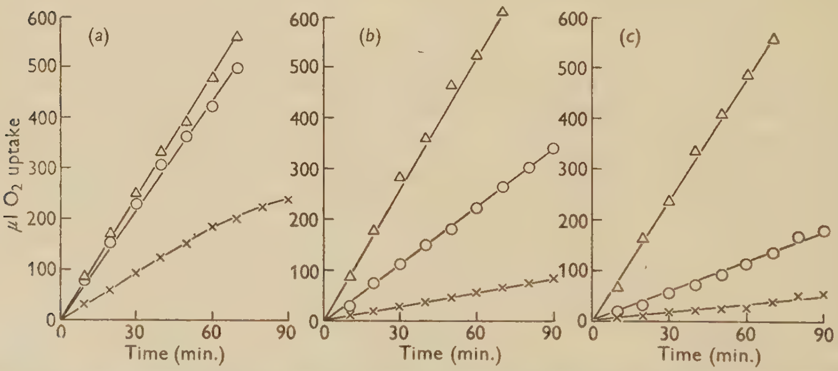

Fig. 2. Oxygen uptake by disrupted bacterial preparations of *Mycobacterium smegmatis*. (*a*) Lemco-grown (6 mg. dry wt./ml.); (*b*) acetate-grown (3·2 mg. dry wt./ml.); (*c*) fumarate-grown (4·1 mg. dry wt./ml.). ×—×, no substrate; ○—○, acetate; △—△, fumarate.

The whole organisms were compared with preparations which had been disrupted in the press. Some of the enzymes of the tricarboxylic acid cycle were found by Millman & Youmans (1955) to be associated with the particulate fractions of mycobacterial extracts. With our disrupted bacterial preparations about 70 % of the acetate-oxidizing system was removed by centrifugation at 10,000 g for 10 min., so for these studies we used the completely disrupted bacterial preparation without removing cell debris or remaining whole organisms. Fig. 2 (*a*–*c*) shows that acetate and fumarate were oxidized at a constant rate by disrupted bacterial preparations from organisms grown on all three media. The rate of fumarate oxidation was always higher than the rate of acetate oxidation, irrespective of the oxidative activity of the corresponding whole organisms. Chloramphenicol had no effect on the rate of fumarate oxidation by the disrupted bacterial preparations but there was a slight inhibition of acetate oxidation after 40 min.

Table 1 summarizes the results obtained for Q_{O_2} with acetate and fumarate as substrates by whole organisms and disrupted preparations in the first 60 min. The method of preparation of the disrupted bacteria always resulted in loss of activity and this varied widely with different preparations so that different batches cannot be directly compared. It can be seen, however, from Table 1 that disrupted preparations of organisms grown on Lemco-agar or acetate-agar oxidized fumarate more rapidly than the corresponding whole cells. The acetate-oxidizing activity of disrupted preparations was always found to be less than that of the whole organisms and is possibly more easily damaged by the method used for cell breakage than is the fumarate enzyme.

Table 1. *The oxidation of acetate and fumarate by* Mycobacterium smegmatis

Substrate added to growth medium		No substrate	Acetate	Acetate + chloramphenicol	Fumarate	Fumarate + chloramphenicol
		μl. O_2/mg. bacterial preparation/0–60 min.				
None	Whole bacteria	9·0	77	58	16	9·0
	Disrupted bacteria	9·6	24	—	26	—
Acetate	Whole bacteria	17·1	166	147	44	19
	Disrupted bacteria	6·1	24	—	56	—
Fumarate	Whole bacteria	8·9	67	48	77	77
	Disrupted bacteria	2·7	8·9	—	39	—

DISCUSSION

Several workers have shown that cell-free extracts of pseudomonads oxidize intermediates of the tricarboxylic acid cycle, but that whole organisms may have a lag period before a constant rate of oxidation is reached—unless the substrate has been added to the growth medium. This adaptation of whole organisms is prevented by treatments which inhibit protein synthesis, and has been interpreted as due to the synthesis of specific factors concerned with the permeability of the bacteria to these substrates (Barrett & Kallio, 1953; Kogut & Podoski, 1953; Clarke & Meadow, 1959).

In our experiments, fumarate was oxidized without a lag period by suspensions of *Mycobacterium smegmatis* grown on fumarate and the rate of oxidation was not altered in the presence of chloramphenicol. On the other hand, when the organisms had been grown on Lemco-agar or acetate-agar, there was adaptation to fumarate oxidation during the experimental period, and both the initial rate and the rate in the presence of chloramphenicol were very low.

The disrupted preparations all oxidized fumarate at a constant rate from zero time, but whereas the rates of fumarate oxidation by disrupted preparations from Lemco-grown and acetate-grown organisms were higher than for the corresponding whole cells, the rate of oxidation of fumarate by disrupted preparations from fumarate-grown organisms was always lower than that for the corresponding whole cells.

We interpret the adaptation to fumarate oxidation by Lemco-grown and acetate-grown organisms as due to the synthesis of a specific fumarate permease which would be already present in cells grown on fumarate. Organisms grown on Lemco-agar had an initial rate of fumarate oxidation, and a rate in the presence of chloramphenicol, no higher than the endogenous rate of oxygen uptake, and we suggest that these cells are initially almost completely devoid of fumarate permease. Organisms grown on acetate had a low but significant initial rate of fumarate oxidation; we suggest that some fumarate permease is present enabling them to adapt more rapidly than Lemco-grown organisms to fumarate oxidation. In addition to the induction of a fumarate permease it is also possible that growth on fumarate or acetate increases the amount of oxidative enzymes present in the cell. If this be so, then it is also possible that this additional enzyme may be more easily destroyed by the treatment used to disrupt the bacteria.

The evidence for an adaptive acetate permease is less clear-cut. The rate of oxidation of acetate by acetate-grown organisms was more than double that of Lemco-grown organisms, but this could have been due to increased production of the oxidative enzymes rather than to increased permease production. It is possible to explain the greater adaptation to acetate oxidation shown by washed suspensions of Lemco-grown and fumarate-grown organisms, as due to permease production, but it is also possible that there was an increase in the amount of oxidative enzymes during the experimental period. It seems likely that the presence of acetate in the growth medium is not essential for *Mycobacterium smegmatis* to be permeable to acetate. When acetate is present, the adaptation to acetate oxidation may be due to increase in oxidative enzymes and to increase in permeability. These experiments suggest that the impermeability of mycobacteria cannot be ascribed solely to the high lipid content of the outer layers of the cell and that specific permeability factors should be considered.

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The Propagation and Preservation of *Eperythrozoon coccoides*

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SUMMARY: The propagation of *Eperythrozoon coccoides* in embryonated hen eggs inoculated by the yolk sac and intravenous routes is recorded. Fourteen continuous serial passages were made in the yolk sac and sixteen passages were made after intravenous inoculation into the chick embryo. The mortality among infected embryos was low; splenic enlargement was the only lesion detected. Once the strain had become established, *E. coccoides* was seen in blood smears taken from some eggs after each passage. Yolk sac or blood from infected embryos proved to be infective for mice on each occasion they were examined; 10^{-6} dilutions of yolk sac were also infective. *E. coccoides* was preserved for short periods at −30° in mouse and chick-embryo blood, but not in yolk sac. The organism was also preserved in mouse blood and chick-embryo tissues at −79°. The presence of 10% (v/v) sterile glycerol appeared to increase the stability of *E. coccoides* in chick-embryo tissues stored at −79°.

No reports were found in the literature about the propagation of any species of eperythrozoa in the laboratory. The limited host range of this group of organisms has made their manipulation difficult and satisfactory methods of cultivation and preservation should afford an opportunity to extend our knowledge of them. This report records the successful propagation of *Eperythrozoon coccoides* in embryonated eggs inoculated by the yolk sac and intravenous routes, and the preservation of this organism at −30° and at −79°.

METHODS

The strain of *Eperythrozoon coccoides* Schilling, 1928, used was obtained from mice at the Molteno Institute, University of Cambridge, by courtesy of Dr June Thurston.

Albino mice (*c.* 20 g.) from a strain infected with *Eperythrozoon coccoides* were used to examine the infectivity of the early passages in chick embryos inoculated by the intravenous route. The albino mice were splenectomized and blood smears taken from them for examination on three alternate days in each week for a 10 day period before inoculation, in order to detect carriers. Subsequently, VS mice (from the National Institute for Medical Research, Mill Hill, through the courtesy of Dr A. W. Gledhill) which are normally free from *E. coccoides*, were used for all experiments. Splenectomized VS mice (*c.* 15–20g.) were used to examine the infectivity of the later intravenous passages, and for all the yolk-sac passages; they were also used in the early experiments with preserved materials. Intact VS mice were used for the later experiments with preserved materials and for the titrations. Each mouse received a standard inoculum of 0·5 ml. by intraperitoneal injection. The experimental groups consisted of three inoculated mice and one control mouse. Only two mice were inoculated with the chick embryo blood harvested after each passage, and one control was kept with every six or fewer inoculated mice in the titration and preservation experiments. Blood smears were taken for examination three times weekly. All mice were treated weekly with benzene hexachloride to control ectoparasites.

The techniques used with embryonated eggs were essentially those of Beveridge & Burnett (1946). The eggs were incubated at 35·5°; deaths within 48 hr. of inoculation were ignored. Crystalline sodium penicillin G and streptomycin sulphate were added to all inocula to give final concentrations of 100 i.u. and 100 μg./ml., respectively. The initial inocula consisted of heparinized mouse blood heavily infected with *Eperythrozoon coccoides*. Dead embryos, and viable embryos from the later passages, were dissected. All passages with embryonic material were made immediately after harvesting.

Batches of six embryonated eggs which had been incubated for 6–8 days were inoculated with 0·25 ml. into the yolk sac. An uninoculated egg was kept with each batch. After each passage, blood and yolk-sac smears were obtained from each egg; portions of yolk and yolk sac were pooled and chopped finely with scissors and suspensions were prepared in sterile Craigie mincers (Craigie, 1949). These suspensions, which contained only yolk, yolk sac and added antibiotics, are subsequently referred to as 'yolk sac'. Serial dilutions of infected yolk sac from the twenty-third and twenty-fifth passages were made in heart infusion broth containing 10% (v/v) horse serum, the whole diluted with an equal volume of normal saline. The suspensions were prepared at room temperature immediately after harvesting and mice were inoculated with 1/100 dilutions as soon as possible.

Embryonated eggs which had been incubated for 10–12 days were inoculated intravenously with 0·05 ml. Owing to a varying mortality after inoculation, a standard number of eggs was not used for each passage. At harvesting, the shell membranes were clarified with heparinized phosphate buffered saline (pH 7·2; 20 i.u. heparin/ml.). Blood smears were made from undiluted blood as far as possible. The blood from all the eggs of each passage was collected, with about 2 ml. heparinized saline. Embryonic blood containing heparinized saline, penicillin and streptomycin is subsequently referred to as 'chick-embryo blood'. For control material, blood smears were taken from three batches of ten normal embryonated eggs.

For the preservation experiments, heparinized and defibrinated blood and serum were obtained by bleeding out splenectomized mice heavily infected with *Eperythrozoon coccoides*; infected yolk sac and chick-embryo blood were obtained from the serial passages in eggs. The chick-embryo tissues always contained added antibiotics, and chick-embryo blood also contained the heparinized saline with which it was harvested. The tissues were stored in 5 ml. screw-capped bottles; most of those stored at −79° had a gas-tight seal of wax between the lid and the bottle. When required for use the samples were thawed in warm tap water.

Smears of mouse blood and of chick-embryo tissues were made on clean grease-free slides. The smears were fixed in methanol and were stained whilst inverted upon curved glass staining trays with Giemsa's stain diluted 1/16 with phosphate buffer (pH 7·2). Mouse blood smears were stained for 45 min. and chick-embryo tissues for 3 hr. The smears were rinsed briefly in phosphate buffer after staining and were allowed to dry over an electric lamp.

RESULTS

The survival of Eperythrozoon coccoides *in embryonated eggs inoculated into the yolk sac*

Four groups of four embryonated eggs were inoculated into the yolk sac with *Eperythrozoon coccoides* in heparinized mouse blood. The eggs were harvested after further periods of incubation of 3, 5, 7 and 10 days. Yolk sac from each group of eggs was inoculated into mice. All the embryos survived until they were harvested. All the inoculated mice developed infections of *E. coccoides* after prepatent periods of 2–4 days. None of the control mice became infected. These results suggested that *E. coccoides* might be conveniently passaged in the yolk sacs of embryonated eggs at weekly intervals.

The propagation of Eperythrozoon coccoides *in the yolk sacs of embryonated eggs*

Embryonated eggs were inoculated with mouse blood heavily infected with *Eperythrozoon coccoides*. This blood was obtained from a mouse inoculated with yolk sac from eggs incubated for 7 days in the earlier experiment. Passages were made at weekly intervals. Three mice were inoculated with yolk sac after each passage except the sixth, eighth, eleventh and thirteenth. Only two mice were inoculated after the ninth passage.

Seven days after inoculation with *Eperythrozoon coccoides*, two of the embryos used for the first passage died. Accordingly, the four viable embryos and the two dead ones were harvested separately, and further eggs and mice were inoculated from each group. Yolk sac from the two dead eggs infected mice with *E. coccoides*. The eggs inoculated with similar material survived for 7 days, yolk sac taken from them failed to infect mice. Yolk sac from the four surviving eggs in the first passage infected mice with *E. coccoides* after prepatent periods of 2 days. From this material, 14 continuous serial passages were made at weekly intervals in the yolk sacs of embryonated eggs. Bacterial contamination interrupted the series at the fifteenth passage. Subsequent passages from stored material have brought the total number of passages to 25.

During the 14 passages 96 embryos were inoculated; 10 of these failed to survive for 48 hr. after inoculation. Only 9 of the remaining 86 embryos did not survive until they were required for harvesting. Two embryos died during the first and thirteenth passages, and one embryo died in each of the eighth, ninth, tenth, eleventh and fourteenth passages. All the deaths occurred on the sixth or seventh day after inoculation, except one, which occurred on the fourth day. At the tenth passage 18 eggs were inoculated, and the incubation of 9 embryos which had survived for 7 days after inoculation was continued, but none hatched. None of the 14 uninoculated embryos died during the period permitted for incubation.

Each of the dead embryos had haemorrhagic lesions. Splenic enlargement was present in two of the nine embryos which died before harvesting and in five of the nine embryos whose incubation was prolonged at the tenth passage. Lesions in viable infected embryos were not investigated during the first 10 passages. Eighteen viable infected embryos were examined at the eleventh to fourteenth passages. Variable degrees of splenic enlargement were found in 16 of these embryos, as judged by comparison with the controls.

Blood smears were obtained from 53 embryos inoculated from the fifth passage onwards. Structures morphologically indistinguishable from *Eperythrozoon coccoides* were seen in 44 smears. The organisms were distributed on and between the erythrocytes, so that smears of infected chick-embryo blood closely resembled blood smears from infected mice. Large numbers of organisms were occasionally seen. No structures identifiable as *E. coccoides* were detected in blood smears taken from 11 uninoculated eggs or from 12 hens of the laying flock.

Occasional structures resembling the annular forms of *Eperythrozoon coccoides* were seen in yolk-sac smears. After the eleventh, thirteenth and fourteenth passages large numbers of these structures were seen in smears made from some inoculated eggs. Although similar structures were never seen in large numbers in smears made from uninoculated eggs, the identity of these rings remains uncertain.

Spleen impression smears were obtained from 17 viable infected embryos of the eleventh to the fourteenth passages. In each case annular organisms were detected in the thinner portions of the smears, and occasionally an infected erythrocyte was seen. No association of the organisms and spleen cells was observed. No organisms were detected in spleen smears made from uninoculated embryos.

Twenty-nine mice were inoculated after the various passages. Apart from three mice which died too soon after inoculation for the infection to manifest itself, all the inoculated mice became infected with *Eperythrozoon coccoides* after prepatent periods of 2–4 days; none of the control mice became infected. Yolk sac harvested after the twenty-third and twenty-fifth passages was infective in 10^{-6} dilutions; one of three mice which received a 10^{-8} dilution of twenty-fifth passage material also became infected with *E. coccoides*. The other five mice which received 10^{-8} and 10^{-10} dilutions, and the controls, remained free of infection. In these and in other titrations it was noted that the length of the prepatent periods varied according to the dose of *E. coccoides* received, and corresponded approximately to the logarithm of the dilution used, e.g. 10^{-2} and 10^{-4} dilutions frequently resulted in prepatent periods of 2 and 4 days.

The propagation of Eperythrozoon coccoides *in embryonated eggs inoculated by the intravenous route*

A preliminary experiment showed that *Eperythrozoon coccoides* survived for at least 5 days in embryonated eggs after intravenous inoculation. Accordingly passages at 5-day intervals were undertaken. At the sixth passage some inoculated eggs were harvested after 5 days and passaged, but when it was found that the remaining eggs, harvested after 7 days, were also infected with *E. coccoides*, all subsequent passages were made at weekly intervals. Two mice were inoculated with chick-embryo blood after each of the first six passages, and after each subsequent even numbered passage.

Eperythrozoon coccoides was carried through 16 continuous serial passages in chick embryos inoculated by the intravenous route. During the 16 passages 88 embryos survived for more than 48 hr. after inoculation; only seven of these embryos died before they were required for harvesting. The incubation of eight surviving embryos of the fourteenth passage was prolonged, but none hatched. These embryos, and four others which died 3 and 4 days after inoculation in the ninth, tenth and eleventh passages, were not examined. One embryo died in the thirteenth passage and two in the fifteenth passage, 6 and 7 days after inoculation. These three embryos, and nine viable embryos from the thirteenth, fourteenth and sixteenth passages, were dissected. The three dead embryos and four viable ones had enlarged spleens, but no other lesions were observed.

Eperythrozoon coccoides was detected in 41 of 74 blood smears which were obtained during the sixteen passages. The number of organisms present varied from very few to very many. Eperythrozoa were not seen in smears taken from eggs harvested during the first three passages. A smear from one embryo harvested at the fourth passage contained visible organisms, as did the smears from three of the five embryos harvested at the fifth passage. Subsequently eperythrozoa were detected in smears from most, but not all, of the inoculated embryos. No structures identifiable as *E. coccoides* were seen in blood smears obtained from 30 normal embryos which had been incubated for 18 days.

Eperythrozoa were detected in six spleen smears obtained from viable embryos of the later passages. No association between the organisms and spleen cells was found.

Chick-embryo blood was inoculated into 22 mice. All the mice became infected with *Eperythrozoon coccoides* except one, which died before manifesting the infection. The prepatent periods ranged from 2 to 5 days, but in those mice in which, owing to the system of thrice-weekly bleeding, *E. coccoides* was first detected 5 days after inoculation, the infection was usually well established. The control mice, with one exception, remained free of infection. One blood smear obtained from an uninoculated VS mouse contained *E. coccoides*, but since earlier and subsequent smears did not contain organisms, it was possible that this mouse was not infected.

The viability of Eperythrozoon coccoides *at* −30° *and at* −79°

Viability at −30°. Samples of heparinized and defibrinated blood and serum infected with *Eperythrozoon coccoides* were placed in a refrigerator at −30°. After storage for 8 days each sample was thawed and mice were inoculated. The inoculations were completed within 30 min. and the residual materials were again stored at −30°. After a further 36 days the samples were thawed again and further mice received the following inocula: heparinized blood 0·5 ml., defibrinated blood 0·4 ml. and serum 0·3 ml.

The inocula of heparinized blood, defibrinated blood and serum which had been stored for 8 days infected mice with *Eperythrozoon coccoides* after pre-

Table 1. *The infectivity of* Eperythrozoon coccoides *after storage in mouse blood and chick-embryo materials at* −30°

Inoculum	Duration of storage (days)	Infectivity for mice
Heparinized mouse blood	8	+
Defibrinated mouse blood	8	+
Mouse serum	8	+
Heparinized mouse blood*	44	+
Defibrinated mouse blood*	44	+
Mouse serum*	44	−
Chick-embryo blood (5th passage)	29	+
Chick-embryo blood (6th passage)	71	−
Yolk sac (6th passage)	14	−
Yolk sac (4th passage)	75	−

* Had been once thawed and frozen again whilst stored.
+ = infective; − = not infective.

Table 2. *The infectivity of* Eperythrozoon coccoides *after storage in mouse blood and chick-embryo materials at* −79°

Inoculum	Duration of storage (days)	Infectivity	
		For mice	For chick embryos
Mouse blood + glycerol	12	+	n.t.
Mouse blood	12	+	n.t.
Mouse blood + glycerol	32	+	n.t.
Mouse blood	32	+	n.t.
Chick embryo blood (8th passage) + glycerol	8	+	n.t.
Chick embryo blood (8th passage)	8	+	n.t.
Chick embryo blood (11th passage)	14	+	+
Chick embryo blood (9th passage)	36	−	n.t.
Chick embryo blood (16th passage) + glycerol	19	+	+
Yolk sac (9th passage) + glycerol	8	+	+
Yolk sac (9th passage)	8	+*	−
Yolk sac (12th passage) + glycerol	19	+	+
Yolk sac (13th passage)	12	+	+
Yolk sac (21st passage) + glycerol	49	+	+
Yolk sac (24th passage) + glycerol	92	+	+

* Infected two of three mice inoculated. + = infective; − = not infective; n.t. = not tested.

patent periods of three days. After storage for 44 days, during which time they had once been thawed and frozen again, heparinized blood and defibrinated blood infected mice after prepatent periods of four days. The serum which had been similarly treated was no longer infective. This experiment and four others with chick embryo tissues are summarized in Table 1.

Viability at -79°. The viability of *Eperythrozoon coccoides* was examined with and without the addition of *c.* 10% (w/v) sterile glycerol. Glycerol was added little by little to infected mouse blood, which was then cooled to -79° at a rate of 2° to 3°/min., The requisite amount of glycerol was added directly to chick-embryo tissues, which were then shaken and cooled to -79° in a storage chest containing solid carbon dioxide. The infectivity of stored chick-embryo materials was tested in mice and in embryonated eggs. After passage in eggs, harvested materials were examined for infectivity in mice. These experiments are summarized in Table 2.

DISCUSSION

In his review, Weinman (1944) recorded no conclusive reports of the cultivation of eperythrozoa in bacteriological media or in tissue cultures; he suggested that claims to have cultivated species of the closely related genus *Haemobartonella* should be cautiously assessed. *Eperythrozoon suis* was carried for one passage in the yolk sacs of embryonated eggs (Splitter, 1952), and single passages of *Eperythrozoon coccoides* were also made in the amniotic fluids and yolk sacs of eggs (Derrick *et al.* 1954); in each case the organisms did not survive further passages. Kessler (1942) failed to cultivate *Haemobartonella muris* in the yolk sacs of embryonated eggs, but Vogelsang & Gallo (cited by Weinman, 1944) claimed to have carried this organism for one passage on the chorioallantoic membrane. In the present study, *E. coccoides* was successfully propagated in embryonated eggs inoculated by the yolk sac and intravenous routes. Although eperythrozoa were detected in blood and spleen smears taken from infected embryos, the organisms were not detected with certainty in yolk-sac smears. Yolk sac and chick-embryo blood were always infective for mice, and no change in infectivity was observed during the successive passages. *E. coccoides* was present in 10^{-6} dilutions of the yolk sac from two of the later passages. Gledhill, Dick & Andrewes (1952) and Gledhill, Dick & Niven, 1955), using a similar diluent, found that *E. coccoides* could be passaged in 10^{-8} dilutions of a suspension of mouse liver and spleen.

The successful cultivation of *Eperythrozoon coccoides* in embryonated eggs suggests that these are at present the most suitable media for the cultivation of other species of Eperythrozoon and perhaps of Haemobartonella. Cultivation should facilitate the study of these organisms by obviating the present need to work continually with experimental animals of the host species. In particular, investigations into the site of multiplication of eperythrozoa and certain aspects of their host-parasite relationship might be conducted in chick embryos, where the absence of many external complicating factors would prove advantageous.

Eperythrozoon coccoides and *E. suis* have been preserved for short periods at temperatures near 0° (Weinmann, 1944; Thurston, 1955; Biberstein, Barr, Larrow & Roberts, 1956). *E. suis* survived for at least 31 days in defibrinated blood stored at −32° (Splitter, 1952), and *Haemobartonella muris* survived for at least 11 weeks in defibrinated rat blood after rapid cooling to −79° (Kessler, 1942). The present results indicate that −79° may be the most satisfactory temperature for the storage of *E. coccoides*. Although the organisms sometimes survived storage at −79° in chick-embryo tissues without added glycerol, these inocula were not always infective for mice and embryonated eggs. It would appear, therefore, that the presence of 10 % glycerol is a worthwhile adjunct.

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On the Formation of Acetoin by Acetobacter

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SUMMARY: The formation of acetoin was studied with 44 strains of the genus *Acetobacter*, when growing on a medium containing DL-lactate as the main carbon source. Most of the strains produced only limited amounts of acetoin. However, strains of the species *A. rancens*, *pasteurianum* and *ascendens* converted most of their substrate into acetoin, up to 74 % of the theoretical amount. The influence of the substrate concentration and of the degree of aeration was studied.

The Voges-Proskauer positive substance was isolated from large-scale fermentations, purified and definitely identified as acetoin. It was present chiefly in the laevorotatory form. Young resting cells oxidized D(–)- lactate, L(+)- lactate, sodium pyruvate, acetoin and diacetyl, often nearly to completion. Cell-free extracts synthesized acetoin from pyruvate optimally at about pH 7. More than 99 % of the enzyme activity was located in the soluble enzyme fraction. Thiamin pyrophosphate stimulated CO_2 and acetoin production from pyruvate. Evidence is presented that acetoin may arise both from the acetaldehyde and from the α-acetolactate pathway.

It has been claimed that acetic acid bacteria are able to produce acetoin. Kitasato (1928) demonstrated for the first time that acetone preparations of *Acetobacter ascendens* formed small amounts of acetoin (acetylmethylcarbinol) from pyruvate. Hermann & Neuschull (1932) studied the decomposition of DL-lactate by 13 species of Acetobacter and concluded that all the strains were capable of forming acetoin, *A. gluconicum* excepted. Frateur (1950) noted that some strains of *A. rancens* converted 25 % of calcium lactate into acetoin. Rao (1955) studied the pyruvate decarboxylase in cell-free extracts of *A. suboxydans*; this enzyme also carried out the synthesis of acetoin.

Acetoin formation by acetic acid bacteria has usually been demonstrated by oxidation to diacetyl, but this method is not specific (Juni & Heym, 1956). However, some acetoin was certainly formed since it has been identified by its derivatives. Kitasato (1928) isolated it from culture media as the *p*-nitrophenylosazone (m.p. 314–315°). Hermann & Neuschull (1932) detected acetoin in the distillate from a 3-week-old culture, both as the *p*-nitrophenylosazone (m.p. 308°) and the phenylosazone (m.p. 242°). The discovery of diacetylmethylcarbinol and of acetylbutanediol (Juni & Heym, 1953, 1954), the investigations of these authors on acetoin synthesis, together with the recent advances in the mechanism of pyruvate decomposition, re-opened the question of the identity of the supposed acetoin produced by acetic acid bacteria. The entire problem thus required re-investigation, particularly in view of its physiological, taxonomic and possible industrial importance.

In the present report the formation of acetoin by numerous strains of the genus *Acetobacter* during the aerobic fermentation of DL-lactate has been studied.

METHODS

Bacteria used. Most of the strains of our collection were obtained from Professor T. Wikén and Dr J. W. la Rivière of the Microbiological Laboratory, Technological University, Delft, the Netherlands, and from the Curator of the National Collection of Industrial Bacteria (NCIB), Teddington, England, Some were obtained from Mr I. Mehlman, Department of Food Technology, Davis, California, U.S.A. Dr J. G. Carr, Long Ashton Research Station, Bristol, England, supplied *Acetobacter estunense*. Several of our own isolates were included in the series.

Culture media. Slopes for storage. The strains were grown for 3 days at 30° on slopes containing 10 % glucose, 3 % $CaCO_3$, 1 % yeast extract (Difco), 2·5 % agar and were afterwards stored at 4°. In these conditions the bacteria were viable for about 4–6 weeks. *Acetobacter peroxydans*, *A. paradoxum* and *A. ascendens* were grown for 5 days at 20° on slopes containing beer, and 12 % gelatine, as described before (De Ley, 1958).

The ordinary lactate medium contained per litre of tap water: 125 ml. 0·067 M-KH_2PO_4, 125 ml. 0·067 M-Na_2HPO_4, 2 g. NH_4Cl, 2 g. yeast extract (Difco), 0·25 g. $MgSO_4.7H_2O$, 10 g. DL-lactic acid; 2 mg. bromcresol purple. It was adjusted to pH 6·5 with NH_4OH. The medium described by Rainbow & Mitson (1953), with DL-lactate as sole carbon source was also used. No difference in the rate or final amount of acetoin production was observed between the two media.

The yeast extract + lactate medium, used for species of the suboxydans group only, contained 1 % yeast extract (Difco) and 1 % ammonium lactate. All the liquid media were dispensed in 200 ml. portions in Erlenmeyer flasks of capacity 1 l. and sterilized by heat at 120° for 20 min.

Cultural procedure. The bacteria from one slope were transferred into the medium in the Erlenmeyer flasks, which were incubated at 30° on a shaker. Samples were taken aseptically at the times indicated in the Table 1 and acetoin determined in them.

Large cultures of some of the bacteria of the oxydans group were prepared by growing them for 2–3 days in batches of 6–8 l. in 10 l. carboys which were kept in a water bath at 30°. The cultures were aerated by means of sterile compressed air with a flow rate of approximately 100 l./hr. The cells were harvested by centrifugation and washed twice with 0·01 M-phosphate buffer (pH 6·0).

Analytical methods. Acetoin was determined by the modified Voges-Proskauer (VP) reaction of Westerfeld (1945). Crystalline acetoin dimer was used as a standard. Colour development, as read in the Klett colorimeter (Klett Mfg. Co., New York, U.S.A.) was linear up to 0·35 μmole in 7 ml. of the reaction mixture. Diacetyl was determined by the reaction with urea and hydroxylamine (White, Krampitz & Werkman, 1946). Pure, freshly distilled diacetyl was used as a standard and the extinction read in the Unicam spectrophotometer (Unicam Instruments, Cambridge, England) at 475 mμ. In our hands, results obtained with the method were not very reproducible, 1 μmole of diacetyl giving optical densities varying from 0·74 to 1·00. However, since very little diacetyl was formed in our cultures, the method was satisfactory as an approximation.

A sample of α-acetolactate acetoxyester was kindly provided by Dr E. Juni. The free acid was determined after boiling with N-H_2SO_4 for at least 15 min.; after neutralization of the solution, the acetoin formed was determined by the method described above.

Cell-free extracts were prepared by disrupting a suspension of the bacteria (100 mg. dry wt./ml.) in 0·01 M-phosphate buffer (pH 6), by means of the Raytheon Sonic Oscillator (Raytheon Mfg. Co., Waltham, Mass., U.S.A.). The turbid suspension was centrifuged at 4° at 11,000 *g* for 1 hr., the precipitate discarded and the supernatant fluid used as 'crude extract'. The latter was fractionated by further centrifugation at 100,000 *g* for 2 hr. at 4° into a 'particle' fraction and 'soluble enzymes' (De Ley & Schell, 1959). The preparations were stored at −20° when not in use.

Manometry. The conventional Warburg respirometer was used at 30°. The contents of the vessels will be described below.

RESULTS

Acetoin formation in growing cultures

Preliminary experiments showed that some of our strains produced a strongly VP-positive substance after several days in the above-mentioned media. Thereupon the 44 strains of the collection were inoculated in the 1 l. Erlenmeyer flasks with the appropriate media as described above. Strains of the suboxydans groups were inoculated into the yeast extract + ammonium lactate medium (on which they grow reasonably well) on the assumption that the cells, growing at the expense of the yeast extract, would decompose the lactate without using it as a carbon source. Samples were taken aseptically at the times indicated in Table 1 and both acetoin and diacetyl determined on them. Table 1 includes only a few representative examples of our results. The capacity for acetoin synthesis varied widely within this genus. Acetic acid bacteria of the groups peroxydans (4 strains, representing 2 species), mesoxydans (9 strains, representing 5 species) and suboxydans (4 strains of one species) usually produced only traces of acetoin. Exceptions were *Acetobacter paradoxum* and *A. mesoxydans saccharovorum*. The most potent acetoin-producing strains were members of the oxydans group, in particular *A. rancens*, *A. pasteurianum* (regarded by Frateur, 1950, as a strain of *A. rancens*) and *A. ascendens*. *A. lovaniense* produced also considerable amounts of acetoin, although much more slowly. In some cases, e.g. *A. vini acetati* (also regarded by Frateur, 1950, as a strain of *A. rancens*) 4939, considerable amounts of acetoin were produced in the initial stages, but were decomposed later. In view of the high yield, both isomers of lactate were used for acetoin synthesis.

From the result of Frateur (1950) it can be calculated that his strain of *Acetobacter rancens* converted 63% of the theoretical amount of Ca lactate into acetoin. From the results of Hermann & Neuschull (1932) it can be deduced that *A. rancens* and *A. ascendens* were their best acetoin producers. A recalculation of their results shows that with the former strains 36% and with the latter 31–34% of the theoretical amount had been formed from Na DL-lactate.

The influence of the degree of aeration was studied with our most active strains. Two identical flasks were inoculated with the same strain, one of them was incubated on a shaker, the other one was shaken manually twice a day. The temperature was 30°. Aeration increased the rate of acetoin production appreciably and both cultures finally gave the same yield with times varying from a few days to one week.

Table 1. *The formation of acetoin from* DL-*lactate by a few representative species of* Acetobacter

Shaken cultures at 30°. The results are expressed as mg. acetoin/100 ml. The growth was recorded after 12 days s optical density in a Klett Colorimeter with filter 66. The media are indicated as follows: O (ordinary lactate edium), R (according to Rainbow & Mitson, 1953), YE (yeast extract with ammonium lactate).

		Time in days									
edium	Group and species	1	2	3	4	5	6	7	9	12	Optical density
	Peroxydans group										
O	*A. peroxydans* 8618	1·6	1·2	3·3	—	—	3·0	—	—	—	169
O	*A. paradoxum*	0	11·3	74·4	—	—	91·6	—	—	—	14
	Oxydans group										
R	*A. rancens* 15	—	16·3	151	244	298	—	195	—	—	90
R	*A. rancens* 23	—	27·5	152	250	254	—	200	—	—	129
O	*A. rancens* Bl 30	—	6·4	17·8	152	332	—	332	—	346	208
O	*A. pasteurianum* 11	—	—	—	140	252	361	—	257	—	60
R	*A. acetosum* 2224	—	7·5	13·3	24·7	6·7	—	18·2	—	—	128
R	*A. kützingianum* 3924	8·4	53·5	48·7	31·8	7·5	—	0	—	—	110
R	*A. turbidans* 6424	—	3·5	9·2	2·1	1·1	—	0	—	—	146
R	*A. mobile* 6428	4·6	1·8	2·9	1·0	0·5	—	0	—	—	227
R	*A. vini acetati* 4939	19	60	180	51	—	0	—	—	—	—
R	*A. ascendans* A	20·2	100·8	144	—	120	—	—	—	—	141
O	*A. lovaniense* 8620	—	2·9	49·2	—	—	—	88·4	—	—	—
R	*A. estunense*	—	0	0·5	1·1	0	0	—	0	—	186
	Mesoxydans group										
R	*A. aceti* Ch31	0	0	0	—	—	0	—	0	—	93
R	*A. xylinum* 12	—	—	2·1	6	9·7	12	7·4	—	—	33
R	*A. mesoxydans saccharovorum*	—	—	—	4·3	11·6	—	21	—	32	29
	Suboxydans group										
YE	*A. suboxydans* 26	1	2·3	—	12·6	—	20	20	—	1	—

Since diacetyl also gives a positive VP reaction, it could not be concluded from the above results alone, how far it was responsible for the VP reaction. The odour of diacetyl was hardly noticeable in the cultures. Chemical estimation showed that the highest amount of diacetyl encountered in the culture media was about 120 μg./ml., corresponding to about 4% of the acetoin. Acetoin was not formed in media where either glucose or glycerol was used as the carbon sources in place of lactate. The influence of lactate concentration was studied with *Acetobacter rancens* 23. The results are summarized in Table 2. Calcium DL-lactate was used instead of the ammonium salt, to prevent a possible poisonous effect of an excess of the NH_4 ion. Although the higher concentration gave the smallest % yield, yet the absolute amount of acetoin produced was higher. Since it crystallized out 8% calcium lactate was an unsuitably high concentration.

Table 2. *Influence of the concentration of calcium* DL-*lactate on acetoin formation by growing* Acetobacter rancens *strain* 23

The results are expressed as mg. acetoin/100 ml. Incubation on a shaker at 30°.

% of lactic acid (w/v)	Age of the culture in days				% of the theoretical yield
	2	3	4	6	
1	167	264	—	292	58
2	224	358	360	372	37
5	406	431	525	537	21
8	387	591	892	940	23

Large-scale production of acetoin

A 10 l. carboy with 5 l. of the above ordinary lactate medium, containing 250 g. of DL-lactic acid as calcium salt, was inoculated with 400 ml. of a 48 hr. culture of *Acetobacter rancens* or *A. pasteurianum.* The medium was aerated with compressed sterile air at a flow of 100 l./hr. Colorimetric examination of the medium showed that 33 g. of acetoin accumulated in 5–7 days. The acetoin was extracted with a slight modification of the method of Fulmer, Underkofler & Bantz (1943). The medium was nearly saturated with anhydrous Na_2SO_4 and centrifuged at 4500 *g* for 25 min. An excess of Na_2SO_4 should be avoided, since it may crystallize out before or during centrifugation and an appreciable amount of acetoin may be lost, probably by trapping in the crystals. The efficiency of the continuous ether extraction was followed daily by taking samples from the aqueous solution and determining acetoin on them; 80 hr. (corresponding to 40–70 l. of ether/5 l. of aqueous solution) were required for almost complete extraction. About 1 g. of acetoin remained unextracted. The ether was dried with anhydrous Na_2SO_4 for at least 24 hr., filtered and distilled off. Approximately 35–40 ml. of a yellow-brown viscous liquid remained, showing a very strong VP reaction and containing only traces of diacetyl. This oily residue was then fractionally distilled. The first fraction contained some ether, some diacetyl and water. The fraction boiling at 135°–142·5° was collected and fractionated again at 142·5°. Yield: 27 ml. It was stored at −15° and analysed.

Identification of acetoin

The following criteria were used for the final identification of the purified compound:

(1) It was a nearly colourless liquid, with a rather unpleasant smell. It was soluble to some extent in ether and completely miscible with water.

(2) After standing for several weeks at −20°, it slowly crystallized out as the dimer.

(3) The boiling point was 142·5° (reported values in the literature 142–144°).

(4) The refractive index at 15° was 1·4193 (reported: 1·4192 and 1·4194).

(5) Oxidation to diacetyl. A solution, containing 626 μg./ml., was oxidized to diacetyl with the ferric ion reagent according to Westerfeld (1945). After heating for 30 min. in a stoppered Nicloux tube in a boiling water bath, the mixture was distilled and diacetyl determined. A solution of the same amount of authentic acetoin was subjected to an identical treatment and gave the same optical density.

(6) The compound gave a crystalline semicarbazone with a melting point of 179°–181° (uncorr.) after four crystallizations from a minimal amount of water. The semicarbazone of authentic acetoin melted at 180·5° (uncorr.). The mixed melting point was unchanged. Reported m.p. in the literature: 180°–185°.

(7) A 2:4-dinitrophenylhydrazone was obtained. It decomposed between 304° and 310° (uncorr.). An authentic sample of acetoin-2:4-dinitrophenylhydrazone gave the same result. Reported in the literature: 317°.

Optical activity of acetoin

Acetoin, prepared with *Acetobacter rancens* 23, had an optical rotation of $(\alpha)_D^{20} = -54°$. The pure compound from *A. pasteurianum* 11 gave $(\alpha)_D^{20} = 71°$. The optical rotation of *laevo*-acetoin, prepared with *Aerobacter aerogenes*, filarial homogenates and animal tissues is $(\alpha)_D^{20} = -84° \pm 2°$ (Tanko, Munk & Aboyni, 1940; Berl & Bueding, 1951). One can therefore calculate from our results that the preparation from strain 23 contained 82 % of the *laevo*-form and 18 % of *dextro*-acetoin. The compound, obtained with strain 11 contained 92 % of the *laevo*-isomer and 8 % of *dextro*-isomer. The acetoin formed by yeast carboxylase showed a specific rotation of −64·5° (Juni, 1952*b*). Values of $(\alpha)_D$ between −40° and −103° have been obtained for yeast acetoin (Kobel & Hackenthal, 1941). Acetoin prepared with the wheat germ enzyme gave an optical rotation of $(\alpha)_D^{20} = 36°$ and consisted thus of about 72 % of the *dextro*- and 28 % of the *laevo*-form (Singer & Pensky, 1952).

Reactions with resting organisms

Oxidations by intact organisms in the Warburg respirometer

The results are shown in Table 3. Acetoin-yielding substrates (L(+)- and D(−)-lactates and pyruvate) were almost completely oxidized. The enzyme systems previously described (De Ley & Schell, 1959) in *Acetobacter peroxydans*, were thus also actively functioning here. Acetoin and diacetyl were oxidized by these organisms, but not by older ones, so that, once accumulated, they were not further decomposed.

Anaerobic pyruvate decarboxylase in intact organisms

Pyruvate decarboxylase is supposed to be a constituent of the acetoin-forming system. Its presence was confirmed in several of our strains by manometric measurements. Each Warburg vessel contained 200 mg. wet wt. intact organisms and 10 μmole of sodium pyruvate in a total of 3 ml. 0·0167 M-phosphate buffer (pH 6·2) in an atmosphere of 95% v/v N_2 and 5% v/v CO_2. With *Acetobacter rancens*, strain 23, the reaction ceased after 3 hr., when 0·65 mole CO_2 per mole substrate had been formed. The reaction did not go to completion, perhaps because of the well-known inhibitory effect of acetaldehyde on the pyruvate decarboxylase. In the presence of 24 mg. of dimedon (a trapping agent for $CH_3.CHO$) per vessel, the theoretical amount of 1·0 mole CO_2 per mole of pyruvate was obtained.

Table 3. *Oxidation of several substrates by resting organisms of three strains of* Acetobacter rancens *in the Warburg respirometer at* 30°

The results are expressed as mole O_2 taken up per mole substrate at the end of the oxidation or after 5 hr. in those cases (marked*) where the reaction was slow. Each Warburg vessel contained 100 mg. wet wt. living bacteria in a final volume of 3 ml. 0·02 M-phosphate buffer pH 6·2. 10 μmole of substrate in the side-arm. 0·1 ml. 15% KOH in the central well.

	Species			
Substrate	*A. vini acetati*	*A. rancens* 23	*A. rancens* 15	Theoretical O_2 uptake
Na D(−)-lactate	2·3*	2·5	—	3
Na L(+)-lactate	2·2	2·2	—	3
Na pyruvate	1·8*	2·3	1·6*	2·5
acetoin	3·9	2·2*	0·3*	5
diacetyl	3·6	1·5*	0·2*	4·5

Reactions with cell-free extracts

These experiments were carried out with *Acetobacter rancens* strain 23.

Biosynthesis of acetoin

Crude extracts contained an active pyruvate decarboxylase (Table 4) and 16·2% of the pyruvate was converted into acetoin. Addition of acetaldehyde increased acetoin considerably. In the presence of dimedon decarboxylation was complete and acetoin synthesis abolished. A similar result was obtained with dialysed soluble enzymes.

Table 4. *Formation of acetoin and* CO_2 *from pyruvate with crude cell-free extracts of* Acetobacter rancens 23

Warburg respirometer at 30°. Each vessel contained 1 ml. of extract, 500 μg. of ThPP, 5 μmole Mg^{++}, 5 μmole Mn^{++} and other additions as indicated below in 3 ml. 0·014 M-phosphate buffer (pH 6·2). Atmosphere: 95% N_2 + 5% CO_2. Acetoin was determined on the digests remaining in the Warburg flasks.

	Vessel no.			
Additions	1	2	3	4
20 μmole Na pyruvate	—	+	+	+
30 μmole CH_3CHO	—	—	—	+
24 mg. dimedon	—	—	+	—
μmole CO_2 produced after 1 hr.	0·8	12·9	21	12·9
μmole acetoin formed	0	1·62	0	4·05

Distribution of acetoin-forming enzymes in the soluble and particulate fractions

The pyruvate decarboxylase and the acetoin-forming enzyme were present in both fractions. However, most of the activity was present in the soluble fraction as shown in Table 5. Since the activity of the soluble enzyme preparation was 9·5 times as high as that of the particles and since the latter were 12 times more concentrated, the total enzyme activity of the soluble preparation was about 114 times greater. The acetoin produced by growing cultures was thus chiefly made by the soluble enzymes and less than 1 % was derived from the particles.

Anaerobically the soluble enzymes converted about 32–36 % of the pyruvate into acetoin. Aerobically they oxidized only about half of the pyruvate and converted about 37 % into acetoin (Table 6). The particles effected the same final results, but much more slowly.

Table 5. *Comparison of the carboligase activity at 20° as between the particulate and the soluble fractions*

The system contained 500 µg. of ThPP, 20 µmole sodium pyruvate, 5 µmole Mg^{++} and the amount of enzyme as indicated below in 3 ml. 0·02 M-phosphate buffer (pH 7·0).

Time (min.)	1 ml. of particles (from 300 mg. of living bacteria)	0·25 ml. of soluble enzymes (from 25 mg. of living bacteria)
	µmole of acetoin formed	
5	0·007	0·065
10	0·014	0·133
20	0·028	0·266

Table 6. *Decarboxylation of pyruvate and formation of acetoin with soluble enzymes from* Acetobacter rancens *strain* 23 *in manometric experiments at* 30°

Each vessel contained 1 ml. of soluble enzymes (from 100 mg. living cells), 500 µg. of ThPP, 5 µmole of Mg^{++}, 5 µmole of Mn^{++} and additions as indicated below in 3 ml. 0·02 M-phosphate buffer pH 6·2. Acetoin was determined on the digests remaining in the Warburg flasks.

	Vessel no.				
	1	2	3	4	5
Content of vessel					
2·6 µmole phenazine	–	–	–	+	+
20 µmole Na pyruvate	–	+	+	+	–
24 mg. dimedon	–	–	+	–	–
KOH in central well	–	–	–	+	+
Gas atmosphere	95 % N_2+5 %CO_2			Air	
µmole CO_2 produced at the end of the reaction (1 hr.)	0·5	12·6	20·3	.	.
µmole O_2 consumed at the end of the reaction (1 hr.)	.	.	.	5·1	0
µmole acetoin formed	0	3·57	0	3·75	0

Influence of thiamin pyrophosphate on the synthesis of acetoin

Thiamin pyrophosphate (ThPP) stimulated both the CO_2 production and the acetoin synthesis. Mg^{++} and Mn^{++} had no noticeable influence. The results are summarized in Table 7. However, when these extracts were dialysed overnight against 0·6 % versene at pH 7·5, they became inactive, unless ThPP and Mg^{++} were added.

Influence of pH value on the acetoin synthesis

Figure 1 shows that the pH optimum was around 7. The enzyme was active over the range pH 5–8.

Table 7. *Influence of* ThPP, Mg^{++} *and* Mn^{++} *on acetoin formation and rate of* CO_2 *production in manometric experiments with gas atmosphere of* 95 % N_2–5 % CO_2 *and at* 30°

Each vessel contained 1 ml. of soluble enzymes (dialysed overnight at 4° against 0·01 M-phosphate buffer (pH 7·0) and several additions as mentioned below in 3 ml. 0·02 M-phosphate buffer (pH 6·2).

	Vessel no.				
	1	2	3	4	5
Content of vessel					
20 μmole Na pyruvate	−	+	+	+	+
500 μg. ThPP	+	+	−	+	−
5 μmole Mg^{++}	+	+	+	−	−
5 μmole Mn^{++}	+	+	+	−	−
μmole CO_2 produced after					
(min.) 5	0	7·4	4·9	6·8	4·9
10	0	9·3	7·4	9·3	7·3
15	0	10·4	9·4	10·5	9·3
70	0	11·0	10·8	11·2	10·7
μmole acetoin formed	0	3·42	1·53	3·42	1·49

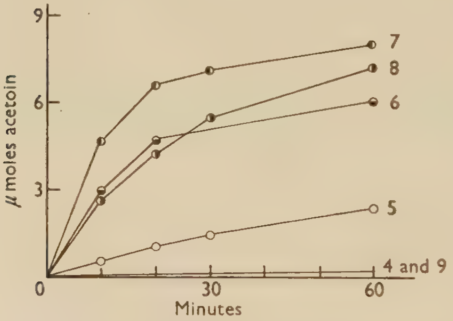

Fig. 1

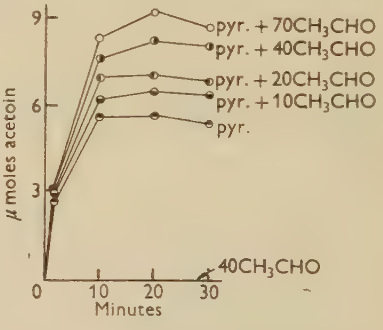

Fig. 2

Fig. 1. pH optimum for the formation of acetoin by cell-free extracts of *Acetobacter rancens*, strain 23, under anaerobic conditions. The reaction mixtures contained in 3 ml. of 0·02 M-buffer, the following ingredients: 500 μg. ThPP, 20 μmole sodium pyruvate and 0·25 ml. of crude extract. The following buffers were used: acetate (pH 4 and 5), phosphate (pH 6 and 7), tris-HCl (pH 8 and 9), NH_4OH-NH_4Cl (pH 10 and 11).

Fig. 2. Influence of acetaldehyde on the formation of acetoin from pyruvate with crude cell-free extracts of *Acetobacter rancens* 23. Reaction mixtures contained in 3 ml. of 0·02 M-phosphate buffer (pH 7·0); 500 μg. of ThPP, 5 μmole Mg^{++}, 20 μmole of sodium pyruvate (pyr.) and varying amounts (μmole) of acetaldehyde. A control with 40 μmole of acetaldehyde alone was included.

Influence of pyruvate concentration on acetoin formation

Table 8 summarizes the results. The yield averaged around 80 % except for the highest concentration, where the reaction was incomplete. This yield was of the same order as that obtained in culture media with growing organisms. The yield in unshaken test tubes was larger than in the Warburg flasks; this difference is ascribed to the fact that by shaking in the Warburg respirometer, part of the CH_3CHO was lost in the gas phase and became unavailable for the reaction.

Table 8. *Influence of pyruvate concentration on acetoin synthesis*

The reaction mixture contained 0·25 ml. of crude extract from *Acetobacter rancens* strain 23, 500 μg. ThPP, 5 μmole of Mg^{++} and various concentrations of Na pyruvate in 3 ml. 0·011 M-phosphate buffer pH 7·0.

μmole Na pyruvate	0	10	20	50	100	160
μmole acetoin formed after (min.) 0	0	0	0	0	0	0
20	0	3·5	7·3	11·6	18·7	12·8
40	0	3·8	7·8	17·8	32·6	27·1
60	0	3·7	8·1	20·4	34·9	34·3
150	0	3·8	8·6	20·4	41·2	44·9
Yield (%)	—	76	86	81	82	(56)

Influence of acetaldehyde on acetoin synthesis

Addition of CH_3CHO to pyruvate in anaerobic conditions in the Warburg respirometer hardly affected the rate of CO_2 production or the total amount of gas formed. It was however repeatedly observed that the amount of acetoin was considerably increased. For example in one experiment 3·42 μmole of acetoin were formed from 20 μmole of pyruvate; in the presence of 20 μmole of pyruvate and 30 μmole of acetaldehyde, 5·79 μmole of acetoin were formed. Figure 2 represents a typical experiment. It thus appears that acetoin can be formed by the enzymic mechanism involving the condensation between acetaldehyde and 'aldehyde-ThPP' (Gunsalus, Horecker & Wood, 1955). This pathway would be similar to the one described in yeast and animal tissues (Juni, 1952*b*). The previous results were obtained with organisms grown on DL-lactate. The acetoin-synthesizing enzyme was constitutive, since preparations from organisms grown on glycerol behaved in the same way and at approximately the same rate.

From acetaldehyde alone, no acetoin was formed, thus indicating that the reaction

$$\text{'aldehyde-ThPP'} + H^+ \rightarrow CH_3CHO + \text{ThPP}$$

was not readily reversed by our enzyme preparations.

Acetoin formation by way of the α-acetolactate pathway

Since there is evidence that our bacteria are able to synthesize acetoin by way of the condensation of acetaldehyde with the 'aldehyde-ThPP' complex, it might be expected that the α-acetolactate pathway would be absent, because the micro-organisms studied so far show either one or the other pathway. Unexpectedly it was found that Acetobacter was not only able to decarboxylate α-acetolactate with the formation of acetoin, but also to produce it from pyruvate. Crude cell-free extracts of *Acetobacter rancens* strain 23, *A. pasteurianum* strain 11, and *A. ascendens* strain A, contained an active α-acetolactate decarboxylase, when grown on either DL-lactate or glycerol as the main carbon source. Figure 3 gives an example of one experiment; 20 μmole of the substrate gave 10 μmole of CO_2, corresponding to complete utilization of one of the isomers. At the end of the experiment, 10 μmole of acetoin were recovered. The amount of α-acetolactate available was too small to determine the configuration of the isomer preferentially attacked. The α-acetolactate decarboxylase activity was about half that of pyruvic decarboxylase in the three strains used.

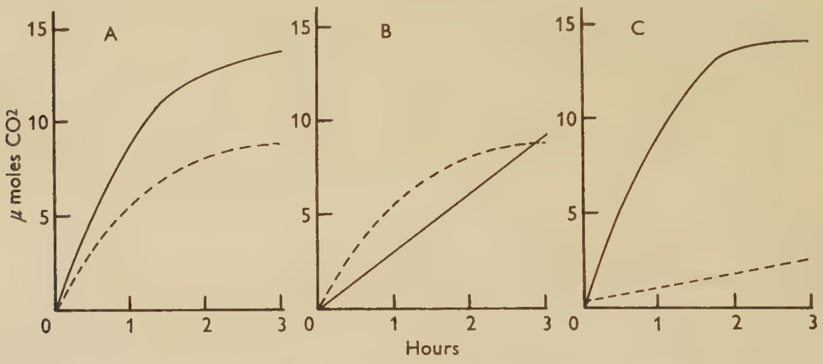

Fig. 3. Presence of α-acetolactate decarboxylase in *Acetobacter rancens* strain 23 and its separation from pyruvate decarboxylase. Each Warburg vessel contained: either 20 μmole Na pyruvate (full lines) or 20 μmole racemic α-acetolactate (broken lines), 500 μg. ThPP, 5 μmole Mg^{++}, 5 μmole Mn^{++} and 1·4 ml. crude extract in 3 ml. 0·028 M-phosphate buffer (pH 6·2). The temperature was 30° and the gas atmosphere 95 % N_2 + 5 % CO_2. In A the crude extract was used, in B after treatment with alumina and in C, after heating.

In an attempt to separate the decarboxylases, two methods described by Juni (1952*a*) for the separation of the *Aerobacter aerogenes* enzymes were used. One sample of the untreated crude extract was used as a control (Fig. 3A). A second sample of 6 ml. of the crude extract was mixed with 1 g. of Microid Polishing Alumina, Grade 3/50, Slow Cutting (Griffin & Tatlock Ltd., London, England), kept at 0° with occasional shaking, centrifuged at 4° and the supernatant was used (Fig. 3B). The α-acetolactate decarboxylase activity of the supernatant remained unchanged, but its pyruvate decarboxylase activity had fallen to 30 % of the original. A third fraction was heated at 70° for exactly 2·5 min., cooled in ice-water, centrifuged at 4° for 10 min. at 10000 r.p.m. and the opalescent supernatant was used (Fig. 3C). The enzymes from our strains behaved quite differently from the Aerobacter enzymes, since in Acetobacter the pyruvate decarboxylase activity was nearly unchanged but the α-acetolactate decarboxylase had been more than 90 % inactivated by the heat treatment. In the experiment, illustrated in Fig. 3C, a distinct odour of acetaldehyde was noticed on opening the vessel with pyruvate and only 0·36 μmole of acetoin had been formed. When an aliquot of the solution was boiled with sulphuric acid, as described in the estimation of α-acetolactate, then neutralized and the acetoin determined, it could be shown that 2·53 μmole of α-acetolactate had been formed, indicating that the enzyme catalysing the reaction:

$$\text{pyruvate} + \text{`aldehyde-ThPP'} \rightarrow \alpha\text{-acetolactate} + \text{ThPP}$$

was present.

Large-scale preparation of acetoin with cell-free extracts

A mixture of 15 ml. 0·067 M-phosphate buffer (pH 7), 15 mg. ThPP, 1·1 g. of sodium pyruvate and 25 ml. of 'soluble enzymes' from *Acetobacter rancens* strain 23, was incubated at room temperature for several hours. The pH, which tended to rise continuously, was maintained at a value of 7. A distinct odour of acetaldehyde was observed. At the end of the reaction, 225 mg. of acetoin was estimated to have been formed. The solution was saturated with sodium sulphate, centrifuged and extracted with ether in a continuous extractor for 25 hr., after which period all the acetoin had passed into the ether. The ether extract was dried with anhydrous Na_2SO_4, filtered and the ether removed by distillation; 170 mg. of an oily, slightly yellowish residue was obtained. The acetoin was converted into the semicarbazone, which, after several crystallizations, melted at the expected value, 180°.

DISCUSSION

Strains of *Acetobacter rancens*, *A. pasteurianum* and *A. ascendens* form considerable amounts of acetoin by a dual pathway. The pyruvate decarboxylase effects the following reactions:

$$\begin{gathered}2\ \text{pyruvate} + 2\ \text{ThPP} \rightarrow 2\ \text{`aldehyde-ThPP'} + 2CO_2\\ \text{`aldehyde-ThPP'} \rightarrow CH_3CHO + \text{ThPP}\\ \text{`aldehyde-ThPP'} + CH_3CHO \rightarrow \text{acetoin} + \text{ThPP.}\end{gathered}$$

This process is identical with that described in yeast and animal tissues (Juni, 1952*b*). It confirms the opinion of Rao (1955) on the mechanism of acetoin formation in *A. suboxydans*. However, our bacteria contain also the enzymes for the formation of acetoin by way of α-acetolactate, according to the reactions:

$$\begin{gathered}\text{`aldehyde-ThPP'} + \text{pyruvate} \rightarrow \alpha\text{-acetolactate} + \text{ThPP}\\ \alpha\text{-acetolactate} \rightarrow \text{acetoin} + CO_2.\end{gathered}$$

This pathway is identical with that found in *Streptococcus faecalis* (Dolin & Gunsalus, 1951) and *Aerobacter aerogenes* (Juni, 1952*a*). It appears thus from our results that Acetobacter is unique in having both pathways, the other micro-organisms in which the biosynthesis has been studied having only one or the other mechanism.

The nearly quantitative recovery of acetoin from the medium shows that it is the main VP-positive substance and that diacetylmethylcarbinol and acetylbutanediol are most probably not formed. The bacteria of the oxydans group appear to be unable to oxidize acetoin to diacetyl and it is also unlikely that appreciable amounts of 2:3-butylene glycol could be formed, in view of the following arguments: (1) we were unable to detect a DPNH-linked reduction of acetoin; (2) the highly oxidizing conditions present during growth.

It has been known for some time that species from the suboxydans and mesoxydans groups are able to produce acetoin from 2:3-butylene glycol. The latter activity should not be confused with the type of metabolism described in the present paper, as it is an illustration of the rule of Bertrand.

The acetoin-synthesizing enzyme is constitutive, since it was also present in cell-free extracts of organisms grown on glycerol. Nevertheless, organism growing on either glycerol or glucose produced no acetoin. This may be taken as an indication that they do not form pyruvate from these substrates and that the glycolytic scheme scarcely functions as a physiological pathway in aerobic conditions. This conclusion is strengthened by previous observations by other authors. It is, for example, known that many acetic acid bacteria, growing on glucose, produce not more than a few per cent of acetic acid (Hermann & Neuschull, 1931). If this be taken as an indication of the operation of the glycolytic pathway, it would mean that only a small portion of glucose is metabolized this way. Furthermore, Kitos, King & Cheldelin (1956, 1957) found that the shunt is much more important than glycolysis in *Acetobacter suboxydans*.

The occurrence of the acetoin-forming enzymes in most of the *Acetobacter rancens* strains and in *A. ascendens* stresses the relationship between both species. According to Frateur, the main difference between these species consists in the fact that *A. ascendens* lacks the capacity to oxidize glucose to gluconic acid. Possibly *A. ascendens* originated from *A. rancens* in the course of evolution by loss of the enzyme system for glucose oxidation. In our collection there were also several strains of *A. rancens* which were barely able to produce acetoin: *A. acetosum* 2224 (*rancens* var. *saccharovorans* according to Frateur), *A. kützingianum* 3924 (*A. rancens* var. *turbidans*), *A. turbidans* 6424 and *A. mobile* 6428 (both *A. rancens*). They are probably the counterparts of *A. ascendens*, which have lost almost completely the capacity to synthesize acetoin, but still retain the one to oxidize glucose to gluconic acid.

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A Note on the Genus *Aeromonas*

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SUMMARY: Previous workers have shown that the flagellation of certain bacteria is variable, becoming lateral or polar according to age and conditions of culture. In view of these findings, and because of the morphological and biochemical similarity of the organisms, Lysenko (1958) has equated *Pseudomonas noctuarum* with *Serratia marcescens*. It is now suggested that *Pseudomonas noctuarum* would be better placed in the genus *Aeromonas*, and that it is similar to an organism provisionally named *A. margarita*. It is further suggested that all members of the genus *Aeromonas* could be regarded as non-chromogenic species of the genus *Serratia*.

Investigation of an epizootic disease which affected laboratory stocks of the desert locust *Schistocerca gregaria* Forsk., resulted in the isolation of a Gram-negative organism which was subsequently shown to be the aetiological agent of the disease (Stevenson, 1958). This organism, initially thought to be a paracolon bacillus (Stevenson, 1954), was later allocated to the group *Aeromonas*, and provisionally labelled *A. margarita*. Recent work by Lysenko (1958) raised doubts regarding both the taxonomic propriety of this classification and the validity of the genus *Aeromonas*. This paper re-examines Lysenko's classification of *Bacillus noctuarum* White in relation to *A. margarita* and the genus *Aeromonas* as a whole.

METHODS

Organisms used. Strains SL10a and SL11a of *Aeromonas margarita* isolated in this laboratory from diseased locusts. Strain 006 of *Pseudomonas noctuarum* (*Serratia marcescens*), from The Laboratory of Insect Pathology, Prague, Czechoslovakia. A strain of *Serratia marcescens* from the London School of Hygiene and Tropical Medicine.

Motility was determined by microscopic examination of hanging-drop preparations after culture in nutrient broth for 2, 4 and 24 hr. at 37°.

Sugar fermentations. Cultures were incubated at 37° in peptone water containing 1·0% (w/v) of the 'sugar', using Andrade's indicator (1·0% acid fuchsin) for the detection of acid production, and Durham tubes for the collection of gas. Examinations were made after 24 and 48 hr. and 5, 10 and 21 days.

Hydrogen sulphide production. Blackening of filter-paper strips, soaked in lead acetate and suspended above nutrient broth enriched with cysteine, was taken to indicate H_2S production. Cultures were incubated at 37° for a maximum of 10 days.

Indole formation. Cultures were incubated in peptone water for 5 days at 37° and tested with Böhmes' reagents.

Urease production was tested on Christensen's (1946) urea agar slopes incubated at 37° for 5 days. A red coloration of the medium was considered to indicate a positive reaction.

Phenylalanine and malonate tests. The medium and methods of Shaw & Clarke (1955) were used.

Gelatin liquefaction was examined at 4° after incubation in nutrient gelatin for 5 days at 37°.

Citrate utilization was tested by inoculating three successive subcultures in Koser's citrate with the tip of a straight wire, and incubating at 37°.

Butanediol production. The organisms were cultured for 48 hr. at 37° in a tryptic meat digest with the addition of 1·0% (w/v) glucose. Butanediol was estimated by Neish's (1952) method and by the method recommended by Pirt (1957).

MR and VP tests. Cultures were incubated in glucose phosphate broth for 5 days at both 30° and 37°, and tested according to the details given by Vaughn, Mitchell & Levine (1939) and by Levine (1941). There was no difference in the results obtained at the different temperatures.

Flagella staining. Loeffler's (1889) and Zettnow's (1899) techniques were used.

RESULTS

All the organisms examined were similar in morphology. They were small Gram-negative rods varying in length from 0·8 to 3·0μ, and in width from 0·8 to 1·0μ. In 24 hr. cultures there was in all cases a preponderance of short, almost coccoid forms. Short rods with bulging sides and longer rods with parallel sides were also present. The ends were rounded and bipolar staining was common.

Motility was observed in hanging drop preparations of all strains, though in *Pseudomonas noctuarum* (*Serratia marcescens*) strain 006 it was apparent only after incubation at room temperature, and not in cultures incubated at 37°. The flagellation of Aeromonas and Serratia strains was apparently polar, though in one or two preparations observed under the light microscope, there were organisms which may have possessed peritrichate flagellation. The only strain examined for flagellation under the electron microscope was *Aeromonas margarita* strain SL10a; in this case all the flagellated organisms were monotrichate. The flagellation of *P. noctuarum* strain 006 was peritrichate.

The biochemical properties of the strains tested are given in Table 1, where they are compared with some of the results obtained by Lysenko (1958). It will be seen that, in general, all the organisms are biochemically similar.

DISCUSSION

Stevenson (1958) allocated the organism isolated from desert locusts to the genus *Aeromonas* on the grounds that it possessed polar flagella and produced butanediol from glucose. As it differed from other organisms listed in the genus, it was provisionally labelled *A. margarita*, though it was pointed out that there are other organisms which resemble it morphologically and in some respects biochemically; these are *Bacillus sphingidis* White (1923*a*), *B. noctuarum* White (1923*b*) and *B. leptinotarsae* White (1928, 1935). These bacilli, though no doubt properly placed in the genus *Bacillus* at the time of their isolation, are now more appropriately placed in the family Pseudomonadaceae, since they are Gram-negative rods with polar flagellation.

Weiser & Lysenko (1956) placed *Bacillus noctuarum* in the genus *Pseudomonas*. However, since it ferments glucose and other sugars, and since *Pseudomonas* by definition excludes fermentative bacteria, it would be better placed in the genus *Aeromonas* were it not VP-negative and therefore not a producer of butanediol (Weiser & Lysenko, 1956). The present tests have confirmed that the organism is VP-positive (Lysenko, 1958). In all probability, therefore, it will produce butanediol and could be an aeromonad. Lysenko (1958), however, allocated it to the genus *Serratia*, and the present work confirms his opinion that strains of *Serratia* tested are, apart from chromogenesis, very similar to Lysenko's strain of *P. noctuarum* (=*Bacillus noctuarum*). In addition, strains of *Aeromonas margarita* are morphologically and biochemically very similar to *P. noctuarum* and to *S. marcescens*.

Table 1. *Biochemical reactions of certain strains of* Serratia marcescens, Pseudomonas noctuarum *and* Aeromonas margarita

	S. marcescens		*P. noctuarum*		*A. margarita*	
	Lko.	Stn.	Lko.	Stn.	Stn.	Stn.
Strain ...			006	006	SL10a	SL11a
Motility	+	+	+	+	+	+
Glucose	d	AG	(G)	AG	AG	AG
Mannitol	AO	AG	AO	AG	AG	Ag
Adonitol	d	nt.	–	nt.	nt.	nt.
Dulcitol	–	–	–	–	–	–
Lactose	×	–	(A)O	–	–	–
Sucrose	AO	AG	AO	AG	A	A
Maltose	AO	AG	(A)O	Ag	A	A
Salicin	AO	AG	AO	Ag	A	A
Inositol	dO	nt.	(A)O	nt.	nt.	nt.
Glycerol	nt.	A	AG	AG	A	A
Gelatin	+	+	+	+	+	+
H_2S	×	+	–	+	+	+
Urease	×	nt.	(+)	nt.	–	–
Indole	–	–	–	–	–	–
MR	–	–	–	–	–	–
VP	+	+	+	+	+	+
Citrates	+	+	(+)	+	+	+
Malonate	–	–	–	–	–	–
Phenylalanine	–	–	–	–	–	–

+ = positive; – = negative; A = acid produced; G = gas produced; g = small quantity of gas produced; O = gas production not tested; × = indeterminate and delayed reaction; d = mutative character (+ or –); ( ) = delayed action; nt. = not tested. Lko. = results obtained by Lysenko (1958); Stn. = results in present paper.

These findings bring into question the validity of the genus *Aeromonas*. First proposed by Kluyver & van Niel (1936) for the bacterium previously known as *Aerobacter liquefasciens* (Beijerinck, 1900), the genus was further studied by Miles & Miles (1951) who came to the conclusion that if the production of butanediol from the fermentation of glucose was a stable character in an otherwise homogeneous group, the genus *Aeromonas* should be accepted. Stanier (1953) confirmed that *A. hydrophila* carried out a butanediol fermentation similar to that of *A. aerogenes* and concurred in its inclusion in the genus *Aeromonas*. The editors of *Bergey's Manual*, 7th edition (1957), list only four members of the genus, but since the preparation of that book, Pivnick & Sabina (1957) have re-examined *Pseudomonas formicans*, isolated by Crawford (1954) from soluble oil emulsions, and have concluded that this organism too, should be included in the genus *Aeromonas*.

An examination of the flagellation of *Aeromonas margarita* leaves no doubt that most of the individuals are mono- or lopho-trichate. However, as pointed out by Lysenko (1958) and Stevenson (1958), the work of Leifson (1951), Leifson & Hugh (1953), Sneath (1956) and others leads to the view that flagellation in the genera *Aeromonas* and *Serratia* may vary according to conditions of culture. Breed (1957) expressed the opinion that such variations have little taxonomic significance, the flagellation of some species having undergone a retrogressive specialization, the organisms becoming primarily dependent on one flagellum as their chief methods of locomotion. Breed said: 'This apparently intermediate type of flagellation seems to be a comparatively recent development...', but produced no evidence for this hypothesis. It may not be so much a recent development, as one that has been recently investigated.

In view of the similarities between the species of *Aeromonas* and *Serratia*, together with the changeable character of the flagellation, it is here proposed that the organism provisionally labelled *A. margarita* is in reality a non-chromogenic strain of *S. marcescens*. Further, it is suggested that all members of the genus *Aeromonas* are unpigmented members of the genus *Serratia*. Obviously, it would be necessary to carry out serological or other studies before such a conclusion could be substantiated; but if it were, it would assist, at least in some degree, to simplify the complicated taxonomy of the Gram-negative bacteria.

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Morphology of the Bifid Bacteria (Organisms Previously Incorrectly Designated *Lactobacillus bifidus*) and Some Related Genera

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SUMMARY: Four morphologically different types of the bifid bacteria can be distinguished, and a classification of these bacteria on morphological basis agrees rather well with earlier groupings based on biochemical and serological characteristics. Morphological features may thus have a taxonomic value as regards the bifid bacteria. A comparison of the morphological characteristics of the bifid bacteria with those of representatives of the genera *Corynebacterium*, *Actinomyces*, *Propionibacterium*, *Butyribacterium* and *Lactobacillus* revealed that three out of four morphological subtypes showed closest resemblance to the genus *Butyribacterium*, whereas the fourth subtype (the one referred to in the literature as *Lactobacillus bifidus* var. *pennsylvanicus*) may be related to *Actinomyces*. Thus, the designation *Lactobacillus bifidus* for these bifid bacteria seems clearly wrong.

The taxonomic relationships of the Gram-positive, anaerobic, asporogenous bacteria of intestinal origin, which are often referred to as the bifid bacteria, seem not to have been clearly defined. Most current schemes of bacterial taxonomy include these organisms in the genus *Lactobacillus* (viz. *L. bifidus*), but strong evidence has been presented to invalidate such classifications (e.g. Negroni & Fischer, 1944; Olsen, 1949; Frank & Skinner, 1954; Hayward, Hale & Bisset, 1955). On the other hand, different proposals for reclassification have been presented, and it has been difficult to decide whether the bifid bacteria are to be transferred to the genus *Corynebacterium*, or to the genus *Actinomyces*, or whether they constitute a distinct group of their own (cf. Prévot, 1938). The problem also serves topical interest, because a number of distinct subtypes of the bifid organisms have been recently described (György, Norris & Rose, 1954; Petuely, 1957; Dehnert, 1957; Gyllenberg & Carlberg, 1958*a*, *b*). Among these the characteristic intestinal inhabitant of breast-fed infants (cf. Petuely, 1957; Gyllenberg & Carlberg, 1958*a*), and the type requiring specific amino sugars as essential nutrients (named *L. bifidus* var. *pennsylvanicus* by György *et al.*, 1954), deserve particular attention.

In spite of the polymorphic nature of the bifid bacteria, morphological characteristics have always been given very great weight in the discussions concerning their taxonomic relationships. In the present study, too, attention was paid to these characteristics, employing the technique of continuous examination of the cultures at different stages of growth, which method has proved successful in a study on *Arthrobacter* (Sundman, 1958).

As already pointed out, the similarity between the bifid bacteria and several other groups of Gram-positive, micro-aerophilic or anaerobic bacteria (particularly corynebacteria and actinomycetes) has been demonstrated by different authors, and, therefore, representatives of these groups, as well as of the genera *Butyribacterium*, *Propionibacterium* and *Lactobacillus*, were included in the study to permit comparison. As regards the corynebacteria, it was assumed that *Corynebacterium acnes* as an obligate to facultatively anaerobic organism is more closely related to the bifid bacteria than are the aerobic corynebacteria. *C. acnes* was selected, therefore, to be the representative of this group.

METHODS

Organisms. Thirteen strains of bifid bacteria were chosen as typical for some hundred isolates from seven faecal samples of breast-, bottle- and spoon-fed infants. The isolates had been obtained, as previously described (Gyllenberg & Carlberg, 1958*a*), by picking all colonies from suitable dilution plates on a highly selective substrate developed by Petuely (1956). This substrate is described below under 'culture media' as the P-agar. After the purity of the isolates had been checked during a few passages as surface streaks on the more complex G-agar, they were kept as stock cultures in tomato agar stabs. The 13 cultures included in the present study are the same as described by Sundman & af Björksten (1958) and by Gyllenberg & Carlberg (1958*b*): A1, A2, A4, A6, B4, B11, B18, C8, C25, D1, E15, K17, M1. Further, the strain ATCC 11863, received in 1956, was included. The three first-mentioned cultures are lacking in Table 1 of Gyllenberg & Carlberg (1958*b*) where the nutritional demands and the fermentation reactions of the strains are listed, but they are comparable with the strains A6 and E15 in that table. Of the cultures included in the present investigation, the strains D1, K17, and M1 were representative of the characteristic 'breast-fed infants' type', which we refer to as *type A* (cf. Gyllenberg & Carlberg, 1958*a*; Sundman & af Björksten, 1958); the cultures B4, B11, and 11863, again, were identified as *Lactobacillus bifidus* var. *pennsylvanicus* (György *et al.*, 1954), but are referred to as *type C* in this report. The other cultures (A1, A2, A4, A6, E15, B18, C8, and C25) were nutritionally identical but differed in fermentation characteristics in that the five first-mentioned cultures utilized pentoses but not sorbitol, while the three last-mentioned cultures behaved vice versa (cf. Gyllenberg & Carlberg, 1958*b*; Sundman & af Björksten, 1958). We prefer, therefore, to distinguish between these cultures (cf. Dehnert, 1957), and to refer to them as *type* B_1, and *type* B_2, respectively. All our isolates of bifid bacteria were strictly anaerobic, no adaptation to aerobic growth was ever noticed.

The following five cultures of the genus *Propionibacterium* were obtained from the Department of Dairy Science, University of Helsinki, through the courtesy of Dr M. Antila: *P. freudenreichii* NCIB 8095, *P. thoenii* NCIB 5966, *P. zeae* NCIB 8100, *P. pentosaceum* NCIB 5961 and *P. intermedium* NCIB 8071.

Butyribacterium rettgeri was kindly supplied by Dr H. A. Barker, University of California, Berkeley.

Four strains of *Corynebacterium acnes* were investigated, viz. NCTC 737, received in October 1956, the strains R68 and AD, isolated by Gyllenberg (1958) as specific contaminants in cultures of bifid bacteria, and the strain ATCC 11617, received as *Lactobacillus bifidus*, but later identified as *C. acnes*.

Two cultures of *Actinomyces bovis* of human origin—A1 and A5, representatives of serological groups A and B respectively (Slack *et al.*, 1955)—were kindly put at our disposal by Dr J. M. Slack, West Virginia University, Morgantown, U.S.A. Unfortunately the sign A1 is the same as used in this laboratory for one of the bifid strains. To avoid confusion and to make it possible to refer to earlier descriptions of the strains (Slack *et al.*, 1955; Sundman & af Björksten, 1958), the nomenclature is not changed.

The following *Lactobacillus* strains were included: *L. helveticus* strains L1 and L4, from the Department of Dairy Science, University of Helsinki; *L. bulgaricus*, isolated in this laboratory from marketed yoghurt; *L. fermenti*, two strains of which (BS21 and BN) were kindly sent to us by Dr Elisabeth Sharpe, National Institute for Research in Dairying, Reading, and the strain V8 which was isolated in this laboratory from infant faeces.

Culture media. The tomato agar used was the same as described by Sundman & af Björksten (1958). For the propionic acid bacteria this substrate was modified to contain glucose instead of lactose, and referred to as tomato agar with glucose. The G-agar was previously described by Sundman & af Björksten (1958). The ascorbic acid and the lactose of this substrate were sterilized separately by filtration and added immediately before use to the rest of the medium sterilized in the autoclave. The P-agar was prepared in the same way and contained per 100 ml. of final medium: ascorbic acid, 1 g.; lactose, 3·5 g.; K_2HPO_4, 0·5 g.; $(NH_4)_2SO_4$, 0·25 g.; cysteine hydrochloride, 0·04 g.; Ca pantothenate, 50 μg; biotin, 0·5 μg.; N-acetyl-d-glucosamine, 20 mg.; Tween 80, 0·5 ml.; 'salts B' solution, 0·5 ml.; agar, 2·5 g.; pH 6·6. The actinomycetes were grown on the CYE substrate according to Howell & Pine (1956), omitting the powdered $CaCO_3$ and substituting the 5 % (v/v) CO_2 + 95 % (v/v) N_2 atmosphere with CO_2 only. The lactate agar employed for the propionic acid had the following composition: peptone Difco, 2 g.; sodium lactate, 1 g.; yeast extract Difco, 1 g.; agar, 1·5 g.; distilled water, 100 ml.; pH 7·0.

General procedure. For continuous examination of morphology, agar plates were inoculated by spreading a small volume of a suspension of the stock culture (tomato agar stab when not otherwise stated) over the surface. The excess of the suspension was poured off and the plates incubated, according to the demands of the organism in question, either with free access to the air or in an desiccator which was evacuated to 15–20 mm. Hg and subsequently filled with CO_2. After various intervals agar blocks were cut out of the plates, and contact prints made for microscopical examination. When, at later growth stages, the compact cell mass gave contact prints too dense for good preparations, ordinary unfixed smears were examined. Main interest was paid to the shape of the living cells as revealed by the phase contrast microscope in water mounts, and to the site and frequency of cross-septa in stained cell-wall preparations. The micrographs were taken on Adox film R14 Pan with a primary magnification of ×830, using Leitz'microscopical equipment. For phase contrast photography we mostly used 12 sec. exposure; for bright field photography of stained preparations, 5–6 sec.

Staining methods. The long exposure time made it difficult to get sharp pictures of water mounts when the cells were very susceptible to Brownian movement. In those cases the print or smear was mounted in dilute crystal violet, 0·02 % (w/v) water solution. This decreased the movement of the cells, and when examined by aid of the ordinary bright field microscope these crystal violet mounts had much of the advantages of water mounts in phase contrast microscopy, and were decidedly better than ordinary stained smears as to the details of the cell contours and evenness of the preparation.

Several cell-wall staining methods were tried. The ordinary tannic acid–crystal violet method (Robinow, 1946) gave diffuse and unsatisfactory preparations of most strains studied. The stronger crystal violet employed in the method of Webb (1954) gave better results, but the mordanting (1 hour 5 %, w/v, tannic acid) was obviously insufficient for the actinomycetes and some of the bifid bacteria. The phosphomolybdic acid + methyl green method (Hale, 1953), the crystal violet–Congo red method (Chance, 1953) and the direct staining method with Victoria blue (Robinow & Murray, 1953) did not give satisfactory results. After some experiments the following two modifications of the methods mentioned above were found most suitable.

1. Phosphomolybdic acid + crystal violet method. The unfixed print or smear was mordanted in 1 % (w/v) phosphomolybdic acid for 5–10 min., washed in tap water, stained with 0·5 % (w/v) crystal violet for 15 sec., washed in tap water, mounted in water.

2. Tannic acid + crystal violet method. The unfixed print or smear is mordanted in 5 % (w/v) tannic acid for one day, washed in tap water, stained with 0·5 % (w/v) crystal violet for 3 min., washed in tap water, studied unmounted. The latter method has some disadvantages; it is time consuming and the prolonged mordanting tends to give a background on the slide. It was used when method No. 1 failed to give satisfactory preparations.

RESULTS

The bifid bacteria. As previously reported (Sundman & af Björksten, 1958), the morphology of the bifid bacteria when grown on tomato agar—a substrate which, with minor modifications, has often been used for cultivation of bifid bacteria—may be abnormal in spite of good growth. Protoplast-like bladders and very pronounced branchings appear on this substrate. Since branching seems to be prevented when the cultures are grown under nutritionally satisfactory conditions (Sundman & af Björksten, 1958), it is our opinion that the unbranched rod form represents the normal form of growth of these bacteria. Pl. 1, fig. 1 and fig. 2 show the branched and unbranched form, respectively, of strain M1 belonging to the subtype A. Petuely & Eichler (1954) came to the same conclusion, and supposed that the rod forms dominate *in vivo*.

It was the scope of the present investigation to compare the morphology of normally growing cultures of the bifid bacteria. The G agar was hence preferred, particularly since it satisfied the nutritional demands of all strains. The only morphologically distinguishable growth cycle noticed on G-agar was a slight tendency to form longer cells at young growth stages (about 15 hr.). The results reported earlier (Gyllenberg, 1955), describing a pronounced variation in morphology with the age of the culture, could not be confirmed with any one of the 14 strains of bifid bacteria studied (cf. Gyllenberg, 1958). It was found most satisfactory to describe the morphology in well developed cultures (24 hr. or more). Although the morphology of the bifid bacteria was more uniform on G-agar than on the poorer tomato agar, it has to be pointed out that even on the former substrate the morphology of our cultures was rather heterogeneous. The following account of the different subtypes is an attempt to find out the specific characteristics of each type rather than to give a description which covers every organism in the cultures studied.

Three isolates were on physiological grounds identified as type A (Gyllenberg & Carlberg, 1958*b*). They were all morphologically similar, though not offering such evident type-characterizing features as the other types described below. The prevailing form of type A in aged cultures was slightly bent, slender rods, 0·2 to 0·4 by 3 to 7μ, frequently somewhat swollen in one or both ends. A granule, optically more dense than the rest of the plasma, was often discernible in the swollen ends with the aid of the phase contrast microscope (Pl. 1, figs. 2–3). This optically dense granule can be regarded as a characteristic of type A. Branches were uncommon. When occurring, they appeared either as rather long, terminal bifurcations or as lateral, rounded buds (Pl. 1, fig. 4.) The rods were separated (Pl. 1, fig. 5), the cross-walls often asymmetrically located.

All the strains identified as type B_1 formed a morphologically well separated group characterized by thick even rods of 0·7 to 0·9 by 2 to 3μ with very few, bud-like, bifid branchings. Pl. 1, figs. 6–9 illustrate the appearance of type B_1. The organisms were occurring separately or grouped in pairs, unicellular, occasionally divided by one central cross-wall into two equal parts. The bud-like branchings were either real branches or separated by a septum from the rest of the organism (Pl. 7, figs. 8–9).

Type B_2 was represented by three cultures. These strains also showed common characteristics separating them from the other isolates of bifid bacteria studied. The curved rods were rather thick, 0·5 to 0·7 by 2 to 8μ. Branchings were very frequent and mostly located as bilateral, adjacent buds on the middle of the organisms. This resulted in a buffer-like or cross-like appearance (Pl. 2, fig. 10). The ends of the rods were mostly swollen and often square cut in connexion with a curvature at the very end of the rod. These horn-like branchings and rods, observed only in the cultures of type B_2, are seen on Pl. 2, figs. 10–11. The rods were mostly unicellular (Pl. 2, fig. 12).

The three cultures of type C (*Lactobacillus bifidus* var. *pennsylvanicus*) formed a morphologically distinct group. The cultures consisted of two kinds of cells; slender rods and clavate swellings. The rods were about 0·4μ in diameter, varying in length from 3 to 5μ (Pl. 2, fig. 13); the clavate organisms were up to 1·5μ in diameter and often very common. In smears from aged cultures it was easy to find microscope fields where almost every organism was clavate. The swellings were accompanied by a slightly bifid structure of the opposite end of the organism, resulting in a peculiar amphora-like appearance, schematically drawn in Fig. 1*a* (cf. Pl. 2, fig. 14). The amphora-like organisms are single or grouped in pairs as seen in Fig. 1*b*, septated as seen in the figure, and consequently composed of 2 to 4 cells (Pl. 2, fig. 15). These forms were typical for the cultures of type C, less frequent in 11863 than in B4 and B11, but not found in the other types of bifid bacteria. The rod form in the type C cultures was septated (Pl. 2, fig. 16). In an earlier report (Sundman & af Björksten, 1958, Pl. 2, figs. 8–11) four photomicrographs illustrating the morphology of the subtypes of bifid bacteria were published. In order not to increase the illustrations in the present paper, these photomicrographs are not republished, but referred to, as being illustrative of the present topic.

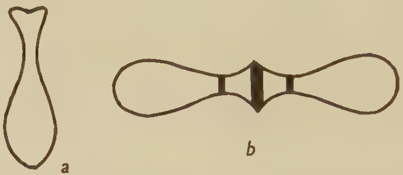

Fig. 1. Schematic presentation of amphora-like organisms found in cultures of type C of the bifid bacteria: (*a*) amphora-like organism; (*b*) peculiar grouping and septation.

The above description of the morphology of the different types of bifid bacteria seems to confirm the earlier mainly physiological classification (Dehnert, 1957; Gyllenberg & Carlberg, 1958*b*). The types B_1 and B_2, corresponding to the groups V and III of Dehnert, respectively, were by Gyllenberg & Carlberg considered as one type named B and characterized by requirements for cysteine (cystine), pantethine (a few strains are capable of growth with pantothenic acid) and riboflavine. The morphological features of the strains separated them in the same distinct groups as Dehnert's classification based on fermentative powers. Our type C (*Lactobacillus bifidus* var. *pennsylvanicus*) corresponds to Dehnert's group I, characterized by rough growth. The smooth form of *L. bifidus* var. *pennsylvanicus* (Dehnert's Group II) was never noticed among our isolates. In Table 1 are collected some data about the four different types of bifid bacteria observed in the present study.

Corynebacterium acnes. This species, found to be the specific 'straight rod' contaminant of cultures of the bifid bacteria (Gyllenberg, 1958), is described as small rods, slightly club-shaped when grown anaerobically. Aerobic conditions are said to induce longer cells and rudimentary branchings (Douglas & Gunter, 1946; *Bergey's Manual*, 1957). All the cultures included in this work formed long branched rods at early growth stages (about 15 hr.) when grown on G-agar in CO_2 atmosphere at 37°. The filamentous rods were 0·2 to 0·4μ in diameter. Later, when the growth had become visible to the naked eye (about 24 hr.), the filaments had broken up into unbranched rods which continually became shorter and after 2–3 days adopted the shape known as typical for anaerobically grown *C. acnes* (Pl. 3, fig. 17). In spite of many attempts with different staining methods we were not able to demonstrate the location of cross-septa in the branched, filamentous growth stages.

Table 1. *Subdivision of the bifid bacteria*

Type (Gyllenberg)	Group (Dehnert)	Basic demands of growth factors*	Characteristic carbohydrate fermentation abilities†	Morphological characteristics on G-agar
A	IV	B, C, Pa	xylose	slender, swollen rods with optically dense granula
B_1	V	C, P, Ri	xylose, arabinose, melezitose	even, stout rods
B_2	III	C, P, Ri	melezitose, sorbitol	branchings, horn-like structures
C	I	C, P, Ri, PPB, As‡	—	1. slender rods 2. amphora-like swellings

* B = biotin, C = cysteine, Pa = pantothenic acid, P = pantethine, Ri = riboflavine, PPB = purine and pyrimidine bases, As = specific amino sugars.

† All strains ferment at least glucose, lactose and saccharose (cf. Dehnert 1957).

‡ Required by freshly isolated strains. Some strains also need a specific peptide factor.

The occurrence of a growth cycle in the cultures of *Corynebacterium acnes* separated this species from all cultures of bifid bacteria included. Even the morphological similarity between, on one side, *C. acnes* at the filamentous and early rod stages and, on the other side, the bifid bacteria, was only superficial. The branched filaments in young *C. acnes* cultures were irregular and of a myceloid appearance not found in the bifid bacteria (Pl. 3, fig. 18). The branches of *C. acnes* rods developed from pointed, lateral buds (Pl. 3, fig. 19), whereas the lateral buds of the bifid bacteria (type A) were rounded (cf. Pl. 1, fig. 4). The bifurcations, seen in all cultures of the bifid bacteria, were never found in the *C. acnes* isolates. Further, *C. acnes* exhibited a constant morphology on G-agar and on tomato agar, whereas the bifid bacteria showed great variability.

Actinomyces bovis. After one subculture in CYE substrate both strains of *A. bovis* were found to contain, beside the filamentous, branched myceloids, also small numbers of cocci. These were supposed to be commensals as described by Erikson & Porteous (1955). Since, nevertheless, the growth on repeated subculture in the semi-solid medium was coherent and filamentous without any turbidity of the substrate, viz. typical for rough *Actinomyces* species, the commensals were assumed not to cause any major disturbance to the host. According to Erikson & Porteous (1955) the fragmentation of *A. bovis* (by these authors named *A. israeli*) may be induced by commensals harboured in small numbers in the Actinomyces culture. The influence is said mainly to cause a variation R → S which in the present work was not the case, since the cultures remained rough and the club-shape, typical for rough actinomycetes, prevailed in aged cultures. Morphologically the two *A. bovis* strains were similar. In young cultures the irregularly branched long filaments prevailed (Pl. 3, fig. 20). With ageing of the culture the filaments divided, and shorter, often club-like fragments developed (Pl. 3, figs. 21–22). The cross-walls developed irregularly, as seen on Pl. 3, fig. 23, where the cells are of various lengths. The club-shaped fragments, seen on Pl. 3, fig. 22, show resemblance to cell forms seen in type C of the bifid bacteria (cf. Pl. 2, fig. 15).

Propionibacterium. The descriptions of the morphology of the propionic acid bacteria in the literature (cf. *Bergey's Manual*, 1957) mostly refer to the work of van Niel (1928). Anaerobic growth is described as circular to short cells, singly, in pairs or in short chains, aerobic growth as irregular club-shaped and branched rods. The 5 strains: *P. freudenreichii*, *P. thoenii*, *P. zeae*, *P. pentosaceum*, *P. intermedium*, included in this study, were all adapted to aerobic growth when received. In aerobically incubated stab cultures in tomato agar with glucose, or on lactate agar the growth was well developed throughout the stab after 1–2 days at 30°. When grown on agar plates with free access to air at room temperature, visible growth developed after 1–3 days. For morphological examination the cultures were grown aerobically in stabs and on plates at 20°, employing both of the substrates mentioned above. *P. thoenii* was also grown on G-agar plates in CO_2 atmosphere at 30°. No striking differences were noticed as a result of these different environmental conditions. Short rods of diphtheroid appearance, oval and pear-shaped cells, single, in pairs and in short chains were found as the prevailing forms in all cultures of *P. freudenrichii*, *P. intermedium* and *P. thoenii*. Young cultures of these strains also contained longer cells, which evidently divided by segmentation into the short elements. Short budlike branches were occasionally found in all three species (Pl. 4, figs. 24–26). *P. pentosaceum* grew as irregular, branched rods, which was the dominating form even after 5 days' incubation in surface cultures as well as in stabs (Pl. 4, fig. 27). *P. zeae* had a distinct growth cycle. After 24 hr. on lactate agar the culture contained irregular rods (Pl. 4, fig. 28) which continuously became shorter during the incubation. After 5 days the culture contained only short oval organisms (Pl. 4, fig. 29). On tomato agar with glucose the segmentation was delayed and the long rods prevailed in stabs and in surface cultures even after 5 days (Pl. 4, fig. 30).

Butyribacterium rettgeri. In the 7th edition of *Bergey's Manual* this species is listed as the single representative for the second genus of the new family Propionibacteriaceae. The morphology is described as follows by Barker & Haas (1944): 'Straight or slightly bent, non-capsulated, non-motile rods, $0{\cdot}7 \times 2\text{–}3\,\mu$, occurring singly, in pairs, and short chains; branched cells not observed but swollen and clubbed cells occasionally formed. Gram positive.' For morphological examination *B. rettgeri* was grown as stab culture in CO_2 atmosphere at 37° in tomato agar, tomato agar with glucose, G-agar and lactate agar. On the two first-mentioned media visible growth developed within 18 hr., on G-agar and on lactate agar somewhat later. The morphology on these latter substrates was in good agreement with the cited description (Pl. 5, fig. 31). On tomato agar and tomato agar with glucose *B. rettgeri* showed a more variable morphology (Pl. 5, fig. 32). The length of the rods varied from 2 to 12μ, branchings were common and of two types: bifurcations as seen on Pl. 5, fig. 32, and asymmetrically located lateral rounded buds (Pl. 5, fig. 33). Cell-wall staining showed that the bifid structures were true branches (Pl. 5, fig. 34), the rods were unicellular or divided by one septum into two cells. The morphological picture was the same throughout the incubation (7 days), and thus the existence of a growth cycle was not indicated.

Lactobacillus. The lactobacilli were grown aerobically on tomato agar either as stabs or as surface cultures. *L. fermenti* (3 strains) was morphologically uniform. The short straight rods consisted of one or two cells as described by Davis, Bisset & Hale (1955); (Pl. 6, fig. 35). The two *L. helveticus* strains contained long rods with a few irregular cross-walls. The rods were often arranged in long curved chains (Pl. 6, fig. 36). This morphology is described as typical for *L. bulgaricus—L. acidophilus* by Davis *et al.* (1955). The *L. bulgaricus* isolate was identified on grounds of lacking volatile acids in the fermentation products of milk cultures, ability to grow at 45°, 48° and 52° but not at 15°, and inability to grow in the presence of 2% NaCl or 2% Na taurocholate (Wheater, 1955). The morphology of this *L. bulgaricus* strain was extremely heterogeneous. In stained preparations the rods varied from short, curved cells in pairs (Pl. 6, fig. 37), similar to those described by Davis *et al.* (1955) as typical for the *L. casei–helveticus* group, to long, sinuous chains (Pl. 6, fig. 38). The short curved rods were fragments of long, twisted spirals, easily detected in water mounts (Pl. 6, fig. 39). As seen in Pl. 6, figs 37 and 40, branched rods were found. The short branchings developed from subterminal or lateral pointed buds, similar to the buds of *Corynebacterium acnes.* Both true and false branchings were seen.

DISCUSSION

The scope of the present investigation was twofold: to settle whether the types of bifid bacteria described by Dehnert (1957) and Gyllenberg & Carlberg (1958*b*), can be identified on a morphological basis, and to find out which of the other groups included, all considered to be related to the bifid bacteria, shows the closest morphological resemblance to these organisms.

As to the first point it can be questioned whether a morphological separation of a group as pleomorphic as that of the bifid bacteria is of any taxonomic value. The ordinary descriptions of cell morphology, including information about dimensions of cells and about cell forms, whether rods or cocci, etc., as generally applied to the genera in the order Eubacteriales, cannot without reservations be used for the bifid bacteria. Because of swellings, variation in cell thickness is so large that figures which cover all the organisms of one subtype at the same time will cover all bifid bacteria. The same is true for the length of the organisms. Further, the morphology shows marked differences on different substrates (cf. Petuely & Eichler, 1954; Dehnert, 1957; Sundman &

af Björksten, 1958). In the present work the complex bifurcations, known from the literature as typical for the bifid bacteria, and illustrated in Pl. 1, fig. 1, were for instance found only on tomato agar among the strains of types A and B_1, whereas the same cultures grew as prevailingly unbranched rods on G-agar. Every morphological description of bifid bacteria should thus be accompanied by a detailed description of the actual cultural conditions. When, however, the morphological examination of the bifid bacteria was performed on cultures grown on a suitable substrate, and the main interest was paid to a few typical configurations and cell forms, in addition to the occurrence of cross-walls, it was found that the morphological differences were big enough to allow a separation.

Our type A of the bifid bacteria (the specific type from breast-fed infants) was not found to possess such striking type-specifying morphological characters as the other types. The optically dense granule seen in the swollen ends of the rods of type A, was not as evident as the stout cell form and the knob-like bifurcations of type B_1, the horn-like branches of type B_2, or the amphora-like organisms of type C. Type A showed morphological resemblances to type B_1 (morphological response to growth on tomato agar) as well as to type B_2 (terminal swellings of the rods) but consisted of septated rods, whereas the organisms of the types B_1 and B_2 were unicellular or contained one central cross-wall. Morphologically the type C (rough form of *Lactobacillus bifidus* var. *pennsylvanicus*) was in several respects different from the other bifid bacteria investigated. The occurrence of slender occasionally filamentous rods, and of club-shaped peculiar amphora-like cells bears resemblance to the morphology of the *Actinomyces bovis* cultures included.

As to the morphological similarity of the bifid bacteria and the related species, the question arises whether all the types of the bifid bacteria should be considered as an entity. The resemblance between the types A and B_1 and *Butyribacterium rettgeri* on one side and between type C and *Actinomyces bovis* on the other side, proved to be at least as close as the internal similarities between these types of the bifid bacteria. The bifurcated rods observed in cultures of *B. rettgeri* on tomato agar were similar to the corresponding forms found in types A and B_1 of the bifid bacteria. The lateral buds seen in *B. rettgeri* were similar in shape and location to those found in type A, isolate K17. The unbranched somewhat irregular rods of *B. rettgeri* were similar in shape to the rod form of type B_1 of the bifid bacteria. A further common feature was the morphological response to the growth environment; the tomato agar induced branchings in the bifid bacteria as well as in *B. rettgeri*. On the other side, the resemblance between the type C and *A. bovis* was not confined to the shape of the cells only. Roughness of growth, poor stainability, and blurred appearance of the cell-wall stained organisms were similar in these cultures. The similarity between the bifid bacteria and the actinomycetes has been pointed out by several authors (e.g. Puntoni, 1937; Negroni & Fischer, 1944; Frank & Skinner, 1954). Unfortunately, their descriptions of the bifid strains in question are not detailed enough to allow the settlement whether type C organisms was dealt with.

The description of *Butyribacterium rettgeri* in the 7th edition of *Bergey's Manual* (1957) obviously needs an addition. The occurrence of branched cells, with true branches as the main structure, was clearly proved in the present work.

As already mentioned in connexion with the description of *Corynebacterium acnes*, the morphological differences between this species and the bifid bacteria seemed to be clear enough to exclude any closer relationship. As a remark on the work of Douglas & Gunter (1946), who placed *C. acnes* in the genus *Propionibacterium*, we wish to state that the morphological criteria found in the present work do not favour such a transfer.

The propionic acid bacteria included in this work formed a morphologically well defined group. The rod forms occasionally found in *Propionibacterium zeae* were similar to rods observed in type B_1 of the bifid bacteria; but the segmentation of the propionic acid bacteria into oval and pear-shaped coccoids is a morphological feature which separates this genus from the bifid bacteria. A common feature of the investigated propionic acid bacteria was the uniform constant thickness of the cells which was independent of the observed variation in length. The bifid bacteria, on the contrary, showed remarkable variations in thickness.

The hitherto unsatisfactory taxonomic position of the bifid bacteria within the genus *Lactobacillus* has given rise to much confusion, and it should be pointed out here that even a superficial microscopical examination will suffice to separate the bifid bacteria from the other lactobacilli. The outer cell walls of the lactobacilli are strictly parallel. Even when strongly curved rods are found, these are throughout even in thickness. The more or less pronounced swellings and irregularities of the rods, always seen in preparations of bifid bacteria, are not found in the lactobacilli. Y- and T-shaped branchings do not *per se* indicate bifid bacteria since, as demonstrated in this report, branching lactobacilli may occur. The differences between lactobacilli and bifid bacteria are most easily seen with the aid of the phase contrast microscope in water mounts or in crystal violet mounts with bright field illumination.

If the taxonomic position of the bifid bacteria is chosen solely on the basis of the morphological relationships within the Gram-positive polymorphic bacteria studied by the present authors, it seems most logical to place them close to the genus *Butyribacterium* (i.e. in the family Propionibacteriaceae as described in the 7th edition of *Bergey's Manual*). As pointed out above, type C (*Lactobacillus bifidus* var. *pennsylvanicus*) makes an exception in this respect and seems to be more closely related to Actinomyces. More information, concerning also physiological and other properties, is needed, however, to permit the decision whether this organism should in fact be separated from the other bifid bacteria.

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EXPLANATION OF PLATES

All plates at ×2500. ph.c. = phase contrast. cr.v.m. = crystal violet mount.

Plate 1

Bifid bacteria of type A (figs. 1–5) and type B_1 (figs. 6–9), grown at 37° in CO_2-atmosphere.

Fig. 1. Strain M1. Branched appearance on tomato agar. 3 days. ph.c.
Fig. 2. Strain M1. Unbranched appearance on G-agar. 3 days. ph.c.
Fig. 3. Strain D1. G-agar. 3 days. ph.c.
Fig. 4. Strain K17. G-agar. 24 hr. ph.c.
Fig. 5. Same as fig. 4. Tannic acid + crystal violet.
Fig. 6. Strain A4. G-agar. 24 hr. ph.c.
Fig. 7. Strain A6. G-agar 3 days. ph.c.
Fig. 8. Strain A2. G-agar. 2 days. Tannic acid + crystal violet.
Fig. 9. Same as fig. 8.

Plate 2

Bifid bacteria of type B_2 (figs. 10–12) and type C (figs. 13–16). Grown on G-agar at 37° in CO_2 atmosphere.

Fig. 10. Strain B18. 4 days. ph.c.
Fig. 11. Strain C25. 4 days. ph.c. Organisms appearing too big, while the water mount tended to dry.
Fig. 12. Strain B18. 24 hr. Tannic acid + crystal violet.
Fig. 13. Strain B4. 24 hr. ph.c.
Fig. 14. As fig. 13. 2 days.
Fig. 15. As fig. 13. Tannic acid + crystal violet.
Fig. 16. As fig. 15.

Plate 3

Corynebacterium acnes (figs. 17–19) and *Actinomyces bovis* (figs. 20–23), grown at 37° in CO_2-atmosphere.

Fig. 17. Strain R68. G-agar. 4 days. ph.c.
Fig. 18. As fig. 17, 15 hr.
Fig. 19. As fig. 18.
Fig. 20. Strain A5, CYE substrate, 24 hr. Phosphomolybdic acid–crystal violet. Commensal micrococci seen at lower right. Note difference in stainability of cell walls of the cocci and those of the actinomycete.
Fig. 21. As fig. 20. 2 days.
Fig. 22. As fig. 20. 5 days.
Fig. 23. As fig. 20. 3 days.

PLATE 4

Propionic acid bacteria. Grown at 20° with free access to the air. cr.v.m.

Fig. 24. *P. freudenreichii.* Lactate agar. 4 days.
Fig. 25. *P. thoenii.* Lactate agar. 3 days.
Fig. 26. *P. intermedium.* Lactate agar. 24 hr.
Fig. 27. *P. pentosaceum.* Lactate agar. 4 days.
Fig. 28. *P. zeae.* Lactate agar. 24 hr.
Fig. 29. As fig. 28. 4 days.
Fig. 30. As fig. 29. Tomato agar.

PLATE 5

Butyribacterium rettgeri grown at 37° in CO_2 atmosphere.

Fig. 31. G-agar. 3 days. cr.v.m.
Fig. 32. Tomato agar. 3 days. cr.v.m.
Fig. 33. Tomato agar. 4 days. cr.v.m.
Fig. 34. As fig. 33. Phosphomolybdic acid–crystal violet.

PLATE 6

Lactobacilli grown at 37° on tomato agar with free access to the air.

Fig. 35. *Lactobacillus fermenti,* strain BS21. 3 days. Phosphomolybdic acid–crystal violet
Fig. 36. *L. helveticus,* strain L4. 3 days. Phosphomolybdic acid–crystal violet.
Fig. 37. *L. bulgaricus.* 2 days. Phosphomolybdic acid–crystal violet.
Fig. 38. As fig. 37. 24 hr.
Fig. 39. As fig. 37. 6 days. cr.v.m.
Fig. 40. As fig. 37.

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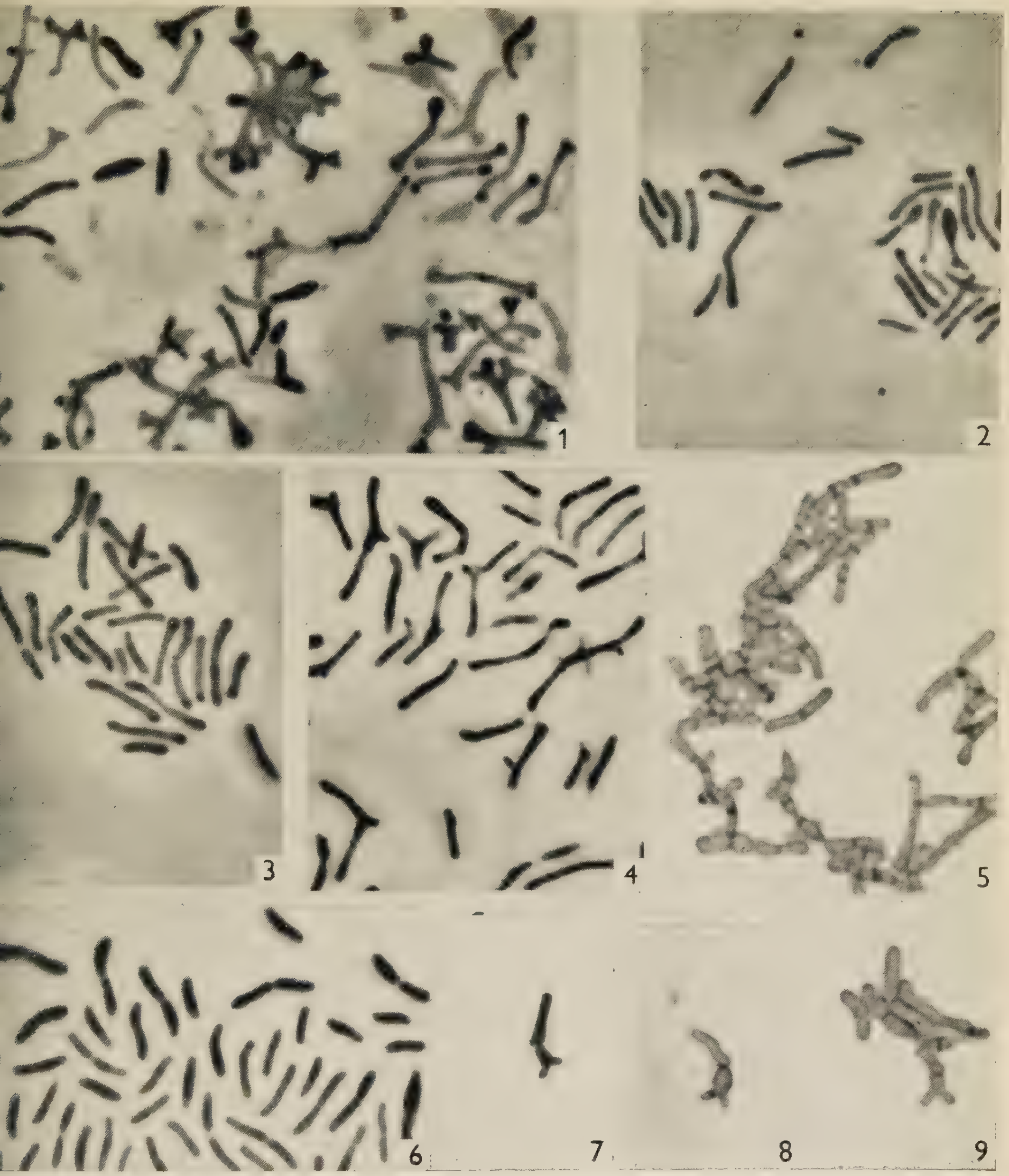

V. Sundman, K. af Björksten & H. G. Gyllenberg—Morphology of bifid bacteria. Plate 1

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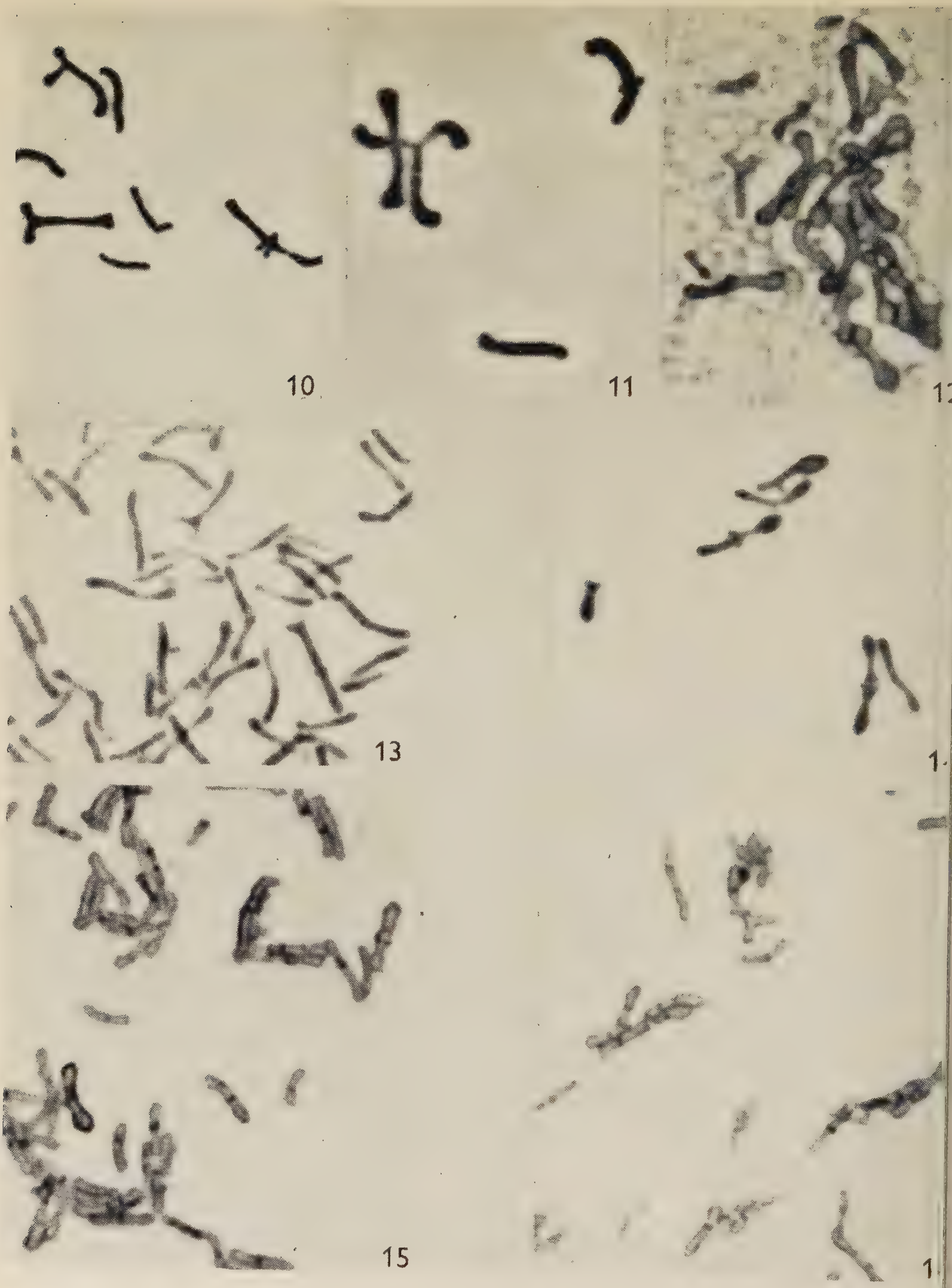
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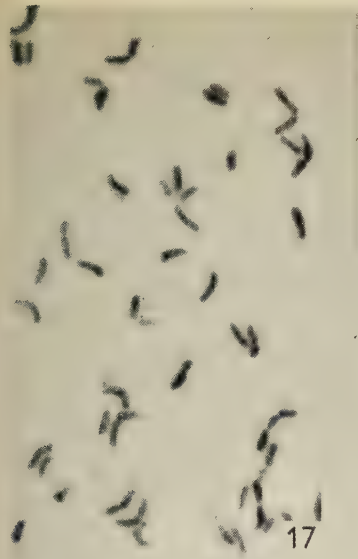
V. Sundman, K. af Björksten & H. G. Gyllenberg—Morphology of bifid bacteria. Plate 2



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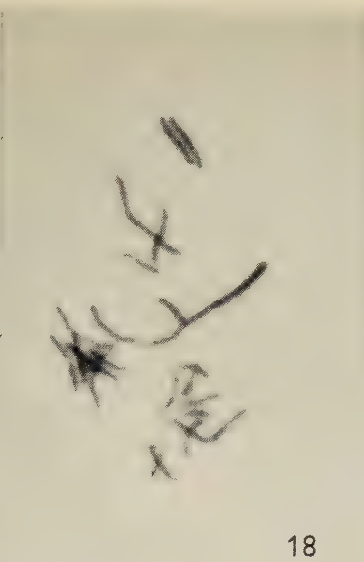

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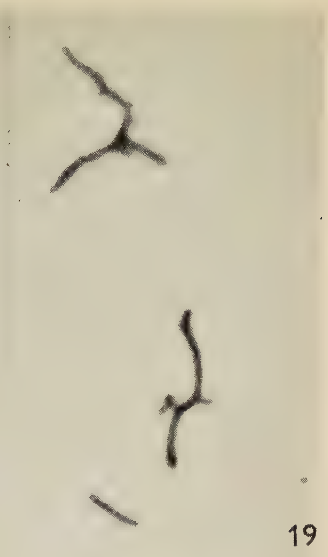

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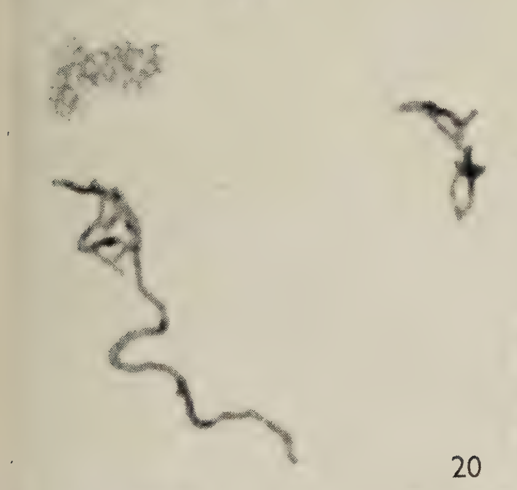

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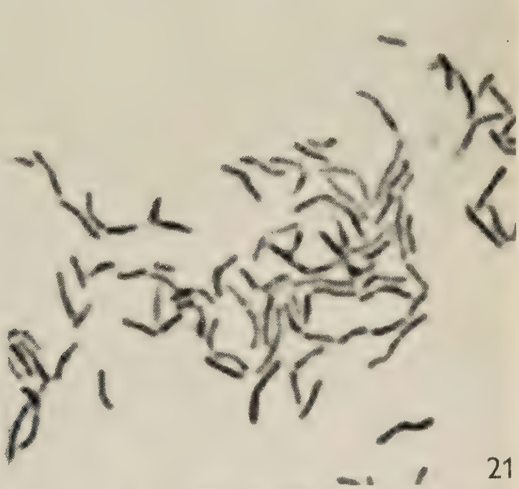

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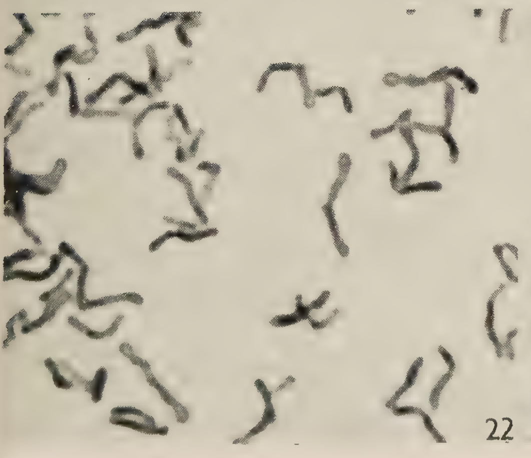

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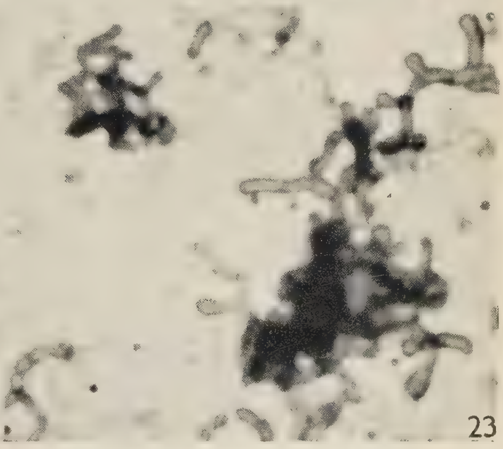

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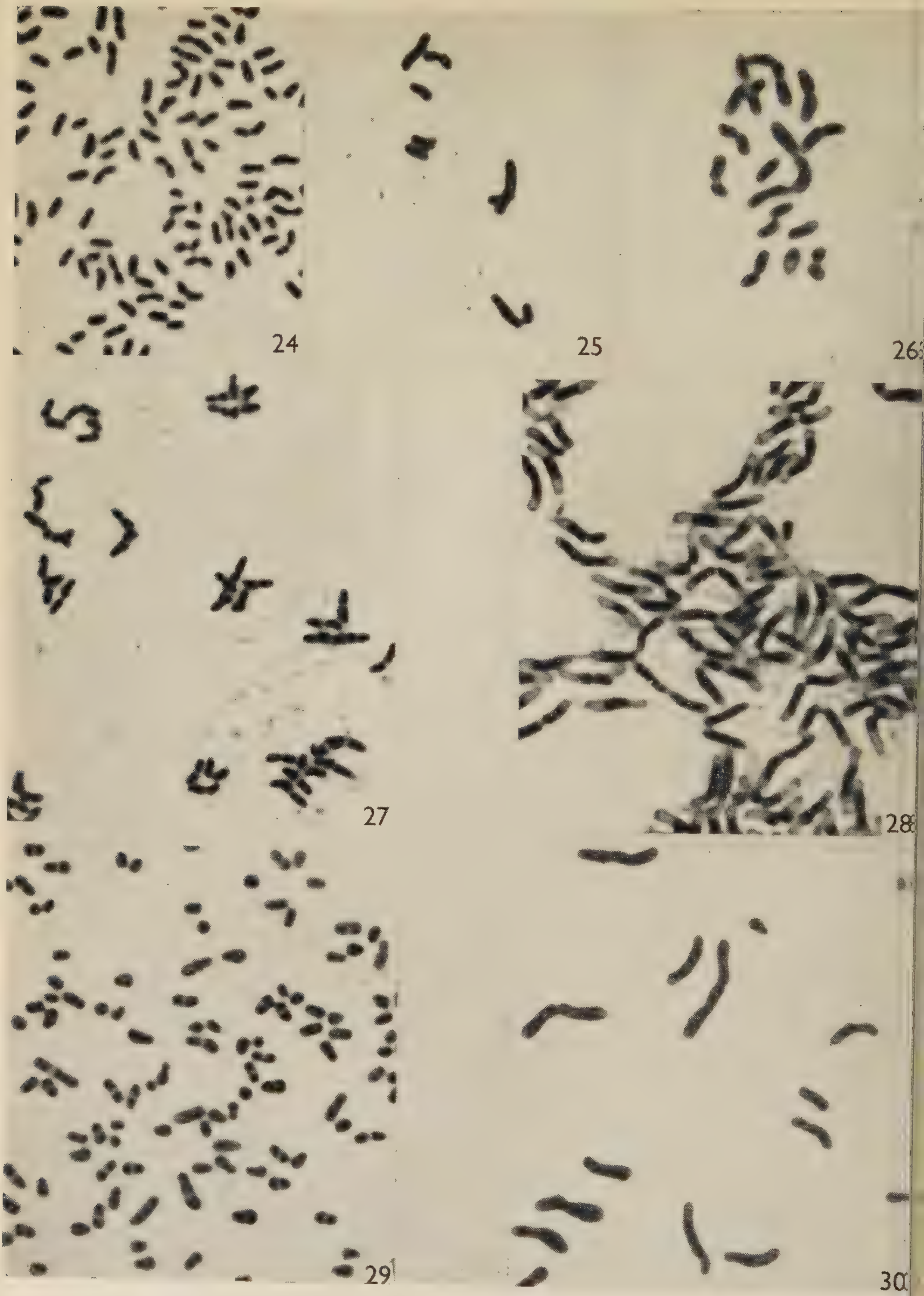
V. SUNDMAN, K. AF BJÖRKSTEN & H. G. GYLLENBERG—MORPHOLOGY OF BIFID BACTERIA. PLATE 3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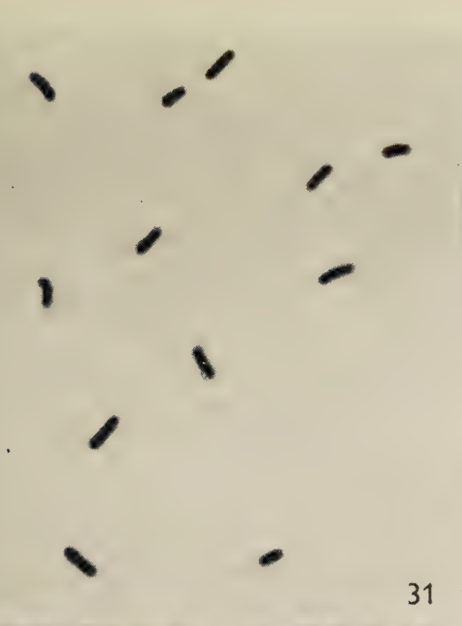

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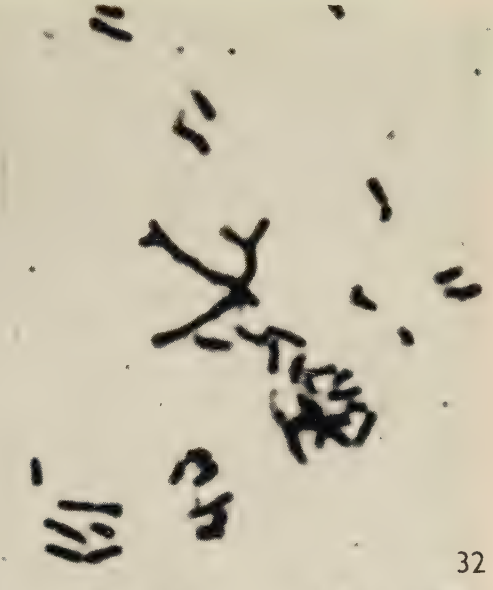

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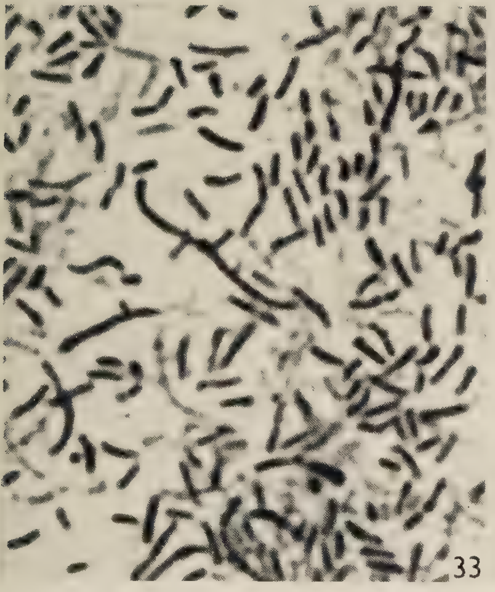

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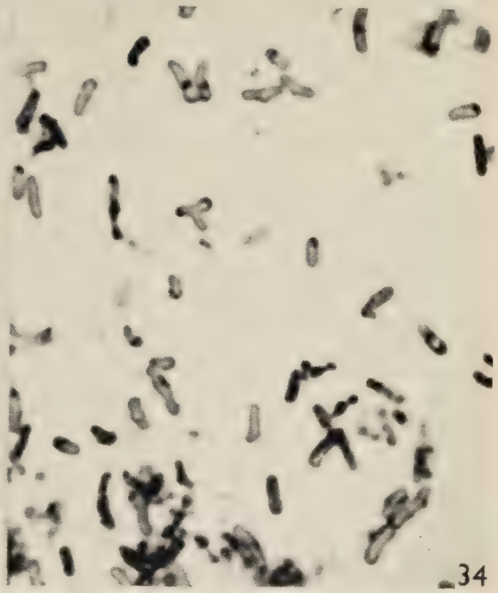

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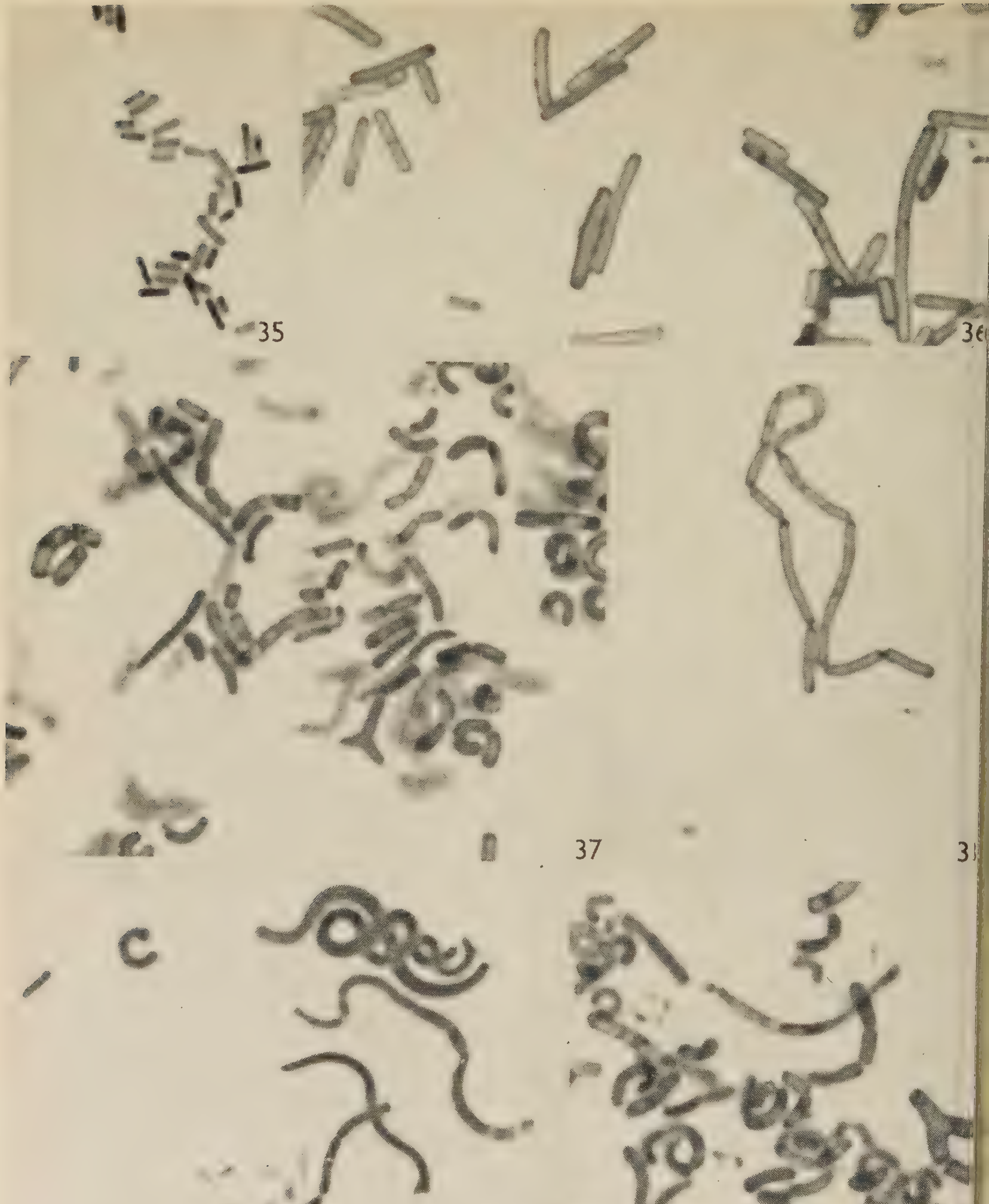

V. Sundman, K. af Björksten & H. G. Gyllenberg—Morphology of bifid Bacteria. Plate 6

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The Clotting of Rabbit Plasma by Group D Streptococci

BY MARION WOOD

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SUMMARY: Some strains of Group D streptococci are capable of coagulating citrated rabbit plasma. This reaction is not due to citrate utilization but to a specific agent elaborated by the organisms; this agent is probably enzymic in nature. The addition of 5 units heparin/ml. to the citrated rabbit plasma inhibits coagulation by streptococci and makes the reaction more specific for staphylococci.

It is now widely recognized that the coagulation of human, rabbit or horse plasma is one of the more reliable diagnostic criteria for the recognition of pathogenic staphylococci. It is also generally inferred that when plasma is clotted as the result of bacterial action, the organism responsible is *Staphylococcus aureus*. But it has been reported that other micro-organisms, particularly Gram-negative coliform organisms, are capable of coagulating plasma (Harper & Conway, 1948; Mushin & Kerr, 1954; Rita, 1954). These workers agreed in concluding that the ability of these bacteria to clot plasma is not due to production of a coagulase enzyme, but to their utilization of the citrate used as the anti-coagulant in the plasma; the release of calcium ion leads to clotting by the normal physiological process. Evans, Buettner & Niven (1952) reported that some Group D streptococci would clot citrated plasma and suggested that this characteristic might, in routine tests used in the food industry, give rise to their diagnosis as late coagulase-positive staphylococci. They also attributed the observed slow coagulation to citrate utilization. Some experiments which invalidate the latter assertion are reported here.

RESULTS

Initial work confirmed the descriptive findings of the American workers, namely that some Group D streptococci would cause plasma coagulation. Citrated rabbit plasma was prepared by mixing equal volumes of a 4 % (w/v) sodium citrate solution and whole blood; thus an approximately $\frac{1}{2}$ dilution of the plasma was obtained in which the citrate concentration was between 2 and 3 %. The inoculum used was 4 drops of a suspension washed from an overnight culture on a blood agar slope by nutrient broth, and this was added to 0·5 ml. of $\frac{1}{2}$, $\frac{1}{6}$ and $\frac{1}{12}$ dilutions of the plasma in saline. The tubes were observed for production of a solid clot after fixed time intervals ($\frac{1}{2}$, 2, 5 hr.) of incubation in a water bath at 37°, and then after 24 hr. at room temperature. Coagulase-positive, late positive and coagulase-negative staphylococcal strains were used as controls and a plasma control with nutrient broth was incubated with each test. About half of the 29 streptococcal strains tested caused plasma to clot within 24 hr. and occasionally within 5 hr.

The organisms tested included *Streptococcus faecalis*, *S. faecalis* var.

liquefaciens, *S. faecalis* var. *zymogenes* and *S. durans*; clotting activity was characteristic of the strain and not of the species.

Citrate utilization. Koser's citrate medium was an unsuitable test medium for the nutritionally demanding organisms being studied and the use of sodium citrate peptone water (Kauffmann, 1954) did not give repeatable results. A chromatographic method proved most satisfactory for detecting citrate under the conditions of the coagulase test.

Samples of citrated plasma subjected to bacterial action, taken at 0, 24 and 48 hr., were macerated when necessary and 8 μl. quantities spotted on to Whatman No. 1 paper. The chromatogram was developed with a tertiary amyl alcohol + formic acid + water mixture (90:20:90 vol.) for 6 hr. The papers were dried and sprayed with a 4 % (w/v) solution of *p*-dimethylaminobenzaldehyde in acetic anhydride. The citrate then appeared as a pink spot, the size and intensity of which gave some indication of the amount of citrate present.

Of those streptococci tested, only one (strain CN 2438) showed evidence of citrate utilization. Other strains which had coagulated plasma within 24 hr., e.g. strains CN 2540 and CN 2538, utilized amounts of citrate insufficient to be detectable by the chromatographic method. In most cases the ionized citrate concentration after 24 hr. appeared to be little changed, and in all instances a final concentration of at least 1 % remained. Clotting could not therefore be due to liberation of calcium ion following destruction of citrate.

Coagulation of decalcified plasma. To confirm that citrate utilization was not the operative factor, plasma stabilized by removing Ca ions with ion exchange resin was used. Rabbit heart blood was pulled through a short column of cation exchange resin (Amberlite 1R/120/H) held in the barrel of the syringe used for withdrawing the blood and then diluted with an equal volume of saline. The blood was centrifuged and plasma stabilized without the addition of anticoagulant was thus obtained. Table 1 shows the results obtained with this plasma and with citrated plasma. The two plasma preparations gave similar results, indicating that citrate metabolism was not responsible for the clotting process.

Coagulation of heparinized plasma. Group D streptococci able to clot citrated plasma, did not clot plasma containing 25 units heparin/ml. At this heparin concentration clotting by late coagulase-positive staphylococci was also delayed. It seemed that heparin might inhibit enzymes responsible for the coagulation phenomenon. Lower concentrations of heparin (5 units/ml.) in citrated plasma effectively eliminated the streptococcal reaction without interfering with the staphylococcal reaction. This observation, of practical diagnostic importance, has not been further investigated.

Extracellular activity. The supposition that streptococci could produce a coagulating enzyme would be confirmed should cell-free preparations be active. Supernatant fluids from centrifuged broth cultures were found to have no detectable activity. However, failure might have been due to poor growth, and other cultural methods were tried. Streptococci were grown inside a cellophan membrane with an external supply of nutrients. Under these conditions better growth was obtained and any extracellular enzymes formed would be retained within the membrane. The centrifuged supernatant fluids from these cultures clotted plasma, with positive reactions in less than 5 hr. in many cases and sometimes in less than 2 hr. The results are summarized in Table 2. Addition of 0·1 % (w/v) sterile bovine albumin solution to cultures within the cellophan bag appeared to increase coagulase activity. Cultures sterilized by filtration were also effective as clotting agents.

Table 1. *The clotting of citrated and decalcified rabbit plasma by Group D streptococci*

	CN	Decalcified plasma									Citrated plasma								
ne (hr.)		½			5			24			½			5			24		
ciprocal of plasma dilution ...		2	6	12	2	6	12	2	6	12	2	6	12	2	6	12	2	6	12
aphylococci																			
oagulase +ve	49	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
oagulase late +ve	69	−	−	−	±	−	−	+	+	+	−	−	−	±	−	−	+	+	+
oagulase −ve	1366	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	±	−	−
oup D streptococci	2536	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	±	±
	2385	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	2538	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	+
	2540	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	+	+
	2530	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	2626	−	−	−	−	−	−	+	+	+	−	−	−	−	−	±	−	+	+
	2438	−	−	−	−	±	+	−	+	−	−	−	−	−	−	−	−	+	+
ntrol		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

+, ± = degree of clotting; − = no clot; CN = strain number.

Table 2. *The clotting of citrated rabbit plasma by supernatant fluid from Group D streptococcal cultures*

	CN	Whole culture									Supernatant fluid								
ne (hr.)		½			5			24			½			5			24		
ciprocal of plasma dilution ...		2	6	12	2	6	12	2	6	12	2	6	12	2	6	12	2	6	12
aphylococci																			
oagulase +ve	49	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
oagulase late +ve	69	−	−	−	+	−	−	+	+	+	−	−	−	+	−	−	+	+	+
oagulase −ve	1366	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
oup D streptococci	2540	−	−	−	−	+	−	+	+	−	−	−	−	−	+	±	−	+	+
	2438	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	+
	2442	−	−	−	−	+	−	+	+	+	−	−	−	−	+	−	−	+	+
	2538	−	−	−	+	+	+	+	+	−	−	−	−	−	+	−	+	+	±
	2530	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	2541	−	−	−	−	+	−	+	+	±	−	−	−	−	+	+	+	+	+
ntrol		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

+, ± = degree of clotting; − = no clot; CN = strain number.

The coagulase activity of the streptococcal filtrate was heat labile. A temperature of 60° for 5 min. had little effect, but heating at this temperature for 15 min. caused clotting to be much delayed; 70° for 15 min. virtually destroyed activity. The preparations were active between pH 5 and pH 9 with little indication of an optimum pH for activity. Nutrient media of pH 8 or slightly above appeared to yield preparations of high activity.

The preparations would not clot solutions of bovine fibrinogen; other plasma factors are involved and the mode of action has not yet been determined.

DISCUSSION

These results show clearly that clotting of plasma by streptococcal filtrates cannot be due to citrate utilization with subsequent clotting by Ca ion as in the normal physiological process. Clotting is due to a specific agent elaborated by the streptococci and this agent is likely to be enzymic in nature. The capacity to produce these enzymes is obviously not limited to the staphylococcus but is more widely distributed. Fredericq (1946 *a*, *b*) reported the production of such reactive agents by *Serratia marcescens* and *Actinomyces* species. Krech (1952) described a cell-free filtrate of *Escherichia coli* which would clot plasma and Billaudelle (1955) listed some strains of *Haemophilus* (*Bordetella*) *pertussis* which had coagulase activity.

It seems unlikely that the streptococcal enzyme functions in precisely the same way as the staphylococcal enzyme, for its action on horse and human plasma does not parallel that on rabbit plasma. This might be because inhibitors which are apparently produced (often there appears to be an optimal concentration) are activated differently in plasmas of the different species. Fractionation of the filtrates would help to resolve this problem.

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Tests for the Rapid Breakdown of Arginine by Bacteria: Their Use in the Identification of Pseudomonads

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SUMMARY: Cultures of 351 miscellaneous Gram-negative rods including 54 pseudomonads were tested for their capacity to break down arginine rapidly by Møller's qualitative technique, by a new qualitative method, and by a quantitative technique which uses standardized washed suspensions. All but 5 of the pseudomonads gave positive results by both qualitative techniques. Among the other organisms tested, positive results were given by Aeromonas strains, some Cloaca strains, some Salmonellas, and, in the case of the new qualitative technique, by 5 strains among other groups. With the quantitative technique 50 % of the arginine was broken down in 2 hr. by all but one of the pseudomonads as compared with only four strains among the other groups. The arginine was broken down completely by 49 of the 54 pseudomonads, but by no other organisms. The ability to break down arginine rapidly is a useful taxonomic feature for pseudomonads and the qualitative tests may have practical value in the identification of strains which do not produce pigment.

During 1958 two concurrent and independent investigations were being made in our laboratories. One concerned the value of tests for amino acid breakdown in the classification of Gram-negative bacilli, and the other was designed to determine whether the phenomenon of arginine-activated motility was limited to pseudomonads (Sherris, Preston & Shoesmith, 1957). The results of these investigations led us to the conclusion that the possession of a potent arginase might be a characteristic useful for the identification of pseudomonads. Further collaborative experiments were therefore made to correlate our findings and to extend the number and variety of the organisms tested. The results of these investigations form the basis of the present communication.

METHODS

Organisms. The strains investigated were all Gram-negative rods. They belonged to a wide variety of genera and species and consisted partly of named cultures from the collection of the Manchester University Department of Bacteriology, the National Collection of Type Cultures, and the National Collection of Plant Pathogens, and partly of strains isolated at the Public Health Laboratory from human material. The strains from the culture collections were accepted as representative of the genera and species to which they had been allocated, and the identity of the freshly-isolated strains was determined as far as possible by morphological, cultural, biochemical and serological examination. All strains classified as pseudomonads were polar flagellate, produced acid from glucose only or no acid from any carbohydrate in 1% peptone water and were strongly oxidase-positive (Gordon & McLeod, 1928; Kovacs, 1956). Most strains also produced blue or green water-soluble pigments and were cytochrome oxidase-positive (Gaby & Hadley, 1957). All but six of the strains were strongly catalase-positive and all but three were insensitive to Erythromycin as judged by absence of inhibition zones around tablets containing 10 μg. of the antibiotic.

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Tests for arginine breakdown

All the strains studied were tested for ability to break down arginine by two qualitative techniques, and some also by a quantitative method.

The qualitative method of Møller (1955). Medium containing (wt./l.): Evans' peptone, 5 g.; Lab Lemco, 5 g.; pyridoxal, 0·005 g.; glucose, 0·5 g.; L-arginine monohydrochloride, 10 g.; bromcresol purple, 0·01 g., and cresol red, 0·005 g., adjusted to pH 6·0, was distributed in narrow tubes to give columns 2 cm. in height. After autoclaving (10 lb./sq.in. for 10 min.), the tubes were sealed with sterile liquid paraffin. A tube of this medium and a control without arginine were inoculated with a straight wire from a nutrient agar culture. Organisms which produced acid from glucose under the conditions of the test rapidly turned the control tube yellow, and those which did not attack arginine also gave a yellow colour in the arginine-containing medium. With organisms, such as pseudomonads, which did not produce acid from glucose in Møller's medium, the control tube remained slate-grey in colour for the first 1–2 days, and sometimes became faintly mauve on further incubation. Arginine breakdown was indicated by the appearance of a violet colour in the tube containing the test medium.

All tests on members of the Enterobacteriaceae were carried out only at 37°; other organisms were tested at 22° and also at 37°. Tubes were examined daily for 5 days. A definite violet colour in the test medium with a yellow, slate-grey or faintly mauve colour in the control tube was taken as evidence of arginine breakdown. After incubation for 4 days the tubes were tested for ammonia with Nessler's reagent as described by Møller (1955).

The Manchester qualitative technique. The workers in the University Department of Bacteriology developed a qualitative test for arginine breakdown, using a medium modified from that described for urease tests by Christensen (1946). It consisted of: peptone (Evans), 1·0 g.; NaCl, 5·0 g.; KH_2PO_4 (anhydrous), 2·0 g.; phenol red, 2·5 ml. of a 0·4% (w/v) solution in water; distilled water to 1 litre. The pH value was adjusted to 6·8, and glucose (1·0 g.) and L-arginine monohydrochloride (10·0 g.) were added. The medium was distributed in 5 ml. amounts in 14 mm. diameter tubes, and autoclaved at 10 lb./sq. in. for 10 min. Two drops (*c.* 0·04 ml.) of a 24-hr. peptone water culture were inoculated into a tube of this medium and into a control without added arginine. The tubes were incubated for 24 hr., in most instances at

37°, but tests on organisms with a lower optimum growth temperature were carried out at 30° or 22°, whichever was appropriate. The colour which developed after incubation was compared visually with that given by buffer solutions of pH 7·0, 7·4, 8·0 and 8·4 containing the same concentration of phenol red as the medium.

The duration of this test and the conditions to be used were determined after preliminary experiments had shown that many organisms, notably strains of Salmonella and Escherichia, produced an alkaline reaction when the time of incubation exceeded 24 hr. Others, such as *Achromobacter anitratus* (*Bacterium anitratum*), would break down arginine under the relatively aerobic conditions obtaining on the surface of a sloped agar-containing medium, but not under the reducing conditions which developed in a deep fluid medium. Breakdown of arginine by pseudomonads, on the other hand, occurred under relatively anaerobic conditions and depended in fluid media on sedimentation of organisms from the area of active growth at the surface. The conditions for arginine breakdown by these strains thus resembled those previously described for *Pseudomonas viscosa* (Sherris *et al.* 1957).

The quantitative arginase reaction. Since the results of the qualitative tests depended on the rate of growth in the test media as well as on the enzymic activity of the organisms, the ability of washed suspensions of organisms to break down arginine under defined conditions was investigated. The organisms investigated included those which gave a positive reaction in 24 hr. by Møller's technique, those which gave a colour change equivalent to pH 7·4 by the Manchester qualitative method, representative samples of each of the genera studied, and all strains of uncertain taxonomic position. Among the salmonellas, however, only the strains which showed the greatest activity against arginine by the Manchester technique were tested. These strains were all positive by Møller's method.

Suspensions were prepared in M/30 Sörensen's phosphate buffer, pH 7·0, from overnight nutrient agar plate cultures. The organisms were washed once and resuspended to an opacity equivalent to Brown's tube No. 10 (equivalent to four times the International Standard of Opacity). Suspension (2 ml.) was mixed in 14 mm. diameter tubes with 2 ml. of mM-L-arginine monohydrochloride in M/30 buffer, and incubated at 37° for 2 hr. A control tube with buffer in place of the arginine solution was treated similarly. Samples (1 ml.) were removed after incubation for 30 and 120 minutes and the organisms lysed by adding 1 ml. of 3N-NaOH. The arginine present was estimated by adding the α-naphthol reagent of Rosenberg, Ennor & Morrison (1956) and measuring the intensity of the colour formed in a Hilger 'Biochem' absorptiometer.

RESULTS

The distribution according to genera or groups of the 351 strains investigated, and the results obtained, are shown in Table 1. The pseudomonads comprised: 28 strains of *Pseudomonas aeruginosa*; 14 strains of *P. fluorescens*; one strain each of *P. graveolens*, *P. mucidolens*, *P. ovalis*, *P. polycolor*, *P. aeruginosa* var. *erythrogenes* and *P. viscosa*; 6 non-pigmented unclassified strains. A positive result by Møller's technique denotes the development of an alkaline reaction as shown by the production of a definite pH indicator change after 24 hr. incubation either at 37° or at 22°. A positive result by the Manchester qualitative technique was taken as the development of a colour change equivalent to pH 8·0 or greater after 24 hr. incubation. A positive reaction by the quantitative technique was taken as the breakdown of over 50 % of the arginine in 2 hr.

Table 1. *Results of tests for arginine breakdown by different bacteria*

		Møller's technique		Manchester technique		Quantitative technique		
Genera or groups	No. of strains	Positive at 24 hr.	Negative at 24 hr.	Positive at 24 hr.	Negative at 24 hr.	Positive	Negative	Not tested
Escherichia	60	0	60	1	59	0	10	50
Shigella	21	0	21	0	21	0	6	15
Klebsiella	28	0	28	0	28	0	7	21
Cloaca	19	7	12	6	13	0	8	11
Serratia	9	0	9	0	9	0	4	5
Citrobacter	10	0	10	0	10	0	6	4
Salmonella	28	24	4	5	23	0	9	19
Hafnia	1	0	1	0	1	0	1	0
Proteus	29	0	29	0	29	0	9	20
Providence	1	0	1	0	1	0	1	0
Pasteurella	3	0	3	0	3	0	3	0
Bordetella	3	0	3	0	3	0	2	1
Achromobacter anitratus (*Bact. anitratum*)	40	0	40	3	37	0	25	15
Achromobacter sp. ('Mima')	21	0	21	0	21	0	21	0
Moraxella	1	0	1	0	1	0	1	0
Alcaligenes	4	0	4	1	3	2	2	0
Vibrio	3	0	3	0	3	0	3	0
Xanthomonas	2	0	2	0	2	0	2	0
Aeromonas	4	4	0	3	1	2	2	0
Pseudomonas	54	50	4	49	5	53	1	0
Unidentified	10	0	10	0	10	0	10	0

Møller's technique. Tests by this method were positive with all but four of the pseudomonads, but with five strains of *Pseudomonas fluorescens*, one of *P. graveolens* and four of the unclassified strains, the positive results were only obtained at 22°. Negative results were given by one strain of *P. fluorescens*, by the strains of *P. ovalis* and *P. mucidolens*, and by one unclassified strain.

Among other genera and groups positive results were given by the four strains of Aeromonas, by the majority of salmonellas and by over half the strains of Cloaca.

The Manchester technique. By this method all but five of the pseudomonads were positive; those which gave negative results were one strain each of *Pseudomonas aeruginosa*, *P. fluorescens*, *P. ovalis*, *P. mucidolens* and one unclassified strain. The strains of *P. aeruginosa* and *P. fluorescens* gave positive results by Møller's method, but the other three were negative by both techniques.

Among other genera and groups positive results were similarly distributed to those given by Møller's method, except that the majority of salmonellas were negative and three strains of *Achromobacter anitratus* (*Bacterium anitratum*), and one each of Escherichia and of Alcaligenes were positive. The discrepancy between the results of the two qualitative techniques with salmonellas was only apparent after 24 hr. incubation. The majority of these strains produced an alkaline reaction in the medium used for the Manchester technique when incubation was prolonged for another day.

The quantitative technique. Among the pseudomonads all but one strain, *Pseudomonas mucidolens*, broke down more than 50 % of the arginine in 2 hr. Among other genera and groups, positive results were also given by two of the aeromonads, and by two strains in the Alcaligenes group. It is of interest that both the positive Alcaligenes strains were polar flagellate, whereas the two negative strains were peritrichate.

When complete breakdown of arginine under the conditions of test is taken as the dividing line between positive and negative results, the test becomes more specific, but less sensitive: 49 of the 54 pseudomonads were positive by this criterion, the exception being two strains of *Pseudomonas fluorescens*, and one strain each of *P. aeruginosa*, *P. ovalis and P. mucidolens*. None of the organisms from other genera and groups brought about complete arginine breakdown.

Unidentified organisms. Ten strains gave negative results by the tests for arginine breakdown. Six of them were non-motile, non-pigment producing rods which were either non-fermenting or glucose-fermenting only. They were either negative or weakly positive by the oxidase and cytochrome-oxidase tests, and differed from pseudomonads in several of their other morphological, biochemical or cultural characteristics. They could not be assigned to the 'Mima' or '*Bacterium anitratum*' groups, and their identity remains in doubt.

Two of the other unidentified strains were also non-motile and fermented a number of carbohydrates without the production of gas; one of them was penicillin sensitive and the other possessed a powerful urease. Once again it was not possible to allocate them with certainty to a particular genus on their general characteristics.

The remaining two unidentified strains were slim, polar flagellate, motile rods. The first produced a yellow non-diffusible pigment, was a facultative anaerobe, gave a negative result by Kovacs' oxidase test after 15 sec., was catalase-negative, and sensitive to Erythromycin. The second strain was non-fermenting, non-pigment producing, oxidase-negative at 15 sec., very weakly catalase-positive and Erythromycin sensitive. Both these strains resembled pseudomonads in their morphology and in some of their biochemical and cultural characteristics. The first of them, however, differed in a number of respects and cannot satisfactorily be included in the genus *Pseudomonas*. The taxonomic relationship of the second strain to *Pseudomonas* is closer, but the combination of poor catalase activity, negative Kovacs' oxidase reaction, and Erythromycin sensitivity set it apart from the other non-pigmented pseudomonads that we studied.

DISCUSSION

The results presented here show that nearly all the pseudomonads tested were able to break down arginine more rapidly than most other Gram-negative rod-shaped organisms, and suggest that this characteristic may prove to be a useful differential feature. It is important to stress that the distinction between pseudomonads and some other bacteria, e.g. Escherichia, in this respect is essentially quantitative and that the specificity of the techniques we have described depends on careful attention to the conditions and duration of the tests.

The quantitative technique proved to be the most satisfactory, because the density of the bacterial suspension and the reaction and composition of the suspending medium could be controlled, while reducing conditions were rapidly established in the reaction mixture. The two qualitative techniques, on the other hand, although giving reasonably similar results, were influenced by a number of uncontrolled factors such as the rate and amount of growth of the organisms, the speed with which oxygen was consumed, and the occurrence of glucose fermentation. These variables account for the somewhat lower specificity of the qualitative tests in detecting those strains with the greatest activity against arginine.

No detailed studies were made to determine the route of breakdown of arginine by the organisms which gave positive results in our tests. It is known that bacteria may act on this substrate either by means of the arginine dihydrolase system (Hills, 1940; Knivett, 1952; Slade & Slamp, 1952) or by means of a decarboxylase. In the dihydrolase system the end products of the breakdown of one molecule of arginine are one molecule of ornithine, two molecules of NH_3 and one of CO_2. This leads to the development of an alkaline reaction and detectable concentrations of ammonia in lightly buffered arginine-containing media such as those used in our qualitative tests. An alkaline reaction may also result from the production of basic amines by decarboxylase activity, but Møller (1955) has shown that this change occurs slowly in his medium, and that a rapid indicator change and the production of NH_3 is the result of arginine dihydrolase activity. All the organisms in our series which gave a positive result at 24 hr. by this technique also showed ammonia production, and thus appear to have possessed the arginine dihydrolase system. Added support for this conclusion has been provided by the direct demonstration of arginine dihydrolase activity in a pseudomonad by Slade, Doughty & Slamp (1954) and also in our strain of *Pseudomonas viscosa* (Shoesmith & Sherris, unpublished observation).

It is of interest that the four strains, other than pseudomonads, which broke down more than 50 % of the arginine in the quantitative test were polarly flagellate. The strains classified as Aeromonas (Kluyver & van Niel, 1936) come from a group of organisms which have, in the past, sometimes been included in the genus *Pseudomonas* (*Bergey's Manual*, 1948), but are considered by more recent workers to merit a separate genus among the Pseudomonadaceae on account of their fermentative capacity and ability to produce 2:3-butanediol from glucose (Stanier, 1943; Miles & Miles, 1951). Our observations show that they have another characteristic in common with pseudomonads in addition to being polarly flagellate. The two positive strains in the Alcaligenes group were also polarly flagellate, and thus conformed to the characteristics of *Vibrio alkaligenes* (see Nyberg, 1935) except that they did not now show any curvature of the rod, and presented few distinguishing features from non-pigment-producing pseudomonads. The possession of polar flagella was not, however, completely correlated with rapid arginine breakdown, as one strain of *V. cholerae* and two of the El Tor vibrio were quite inactive in this respect.

The identification of pseudomonads in practice may, as Gaby & Hadley (1957) have pointed out, present difficulties with strains not producing pigment. The tests for arginine breakdown that we have used may prove to have some practical value in identifying such strains. The qualitative techniques are simple to perform and a positive result after 24 hr. incubation with a strain which does not produce acid from any sugar, or only from glucose, strongly indicates that the organism is a pseudomonad. Of the two methods, that of Møller appears to be a little more specific than the Manchester technique in this respect. The quantitative test is the most specific, and, in our experience, complete arginine breakdown under the conditions described is produced only by members of the genus *Pseudomonas*.

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Lethal Suppressive Cytoplasms in Aged Clones of *Aspergillus glaucus*

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SUMMARY: An irreversible cessation of growth, accompanied by the death of the growing tips and the appearance of an intense brown pigment, is a regular feature of aged clones of *Aspergillus glaucus*. The frequency of this phenomenon, referred to as 'vegetative death', varies between clones of similar age and between identically maintained lineages of the same clone. It also varies with the method of propagation and with the other factors which affect the speed of ageing.

The heterokaryon and related tests show that vegetative death is cytoplasmic in origin while its segregation in the asexual progeny of colonies with mixed cytoplasms, and its infective spread via hyphal anastomoses, clearly define it as mutational in origin. Vegetative death involves a qualitatively different kind of cytoplasmic change from that previously found in the earlier stages of ageing.

In common with some other cytoplasmic mutants, those responsible for vegetative death suppress their wild type counterparts in cytoplasmic mixtures even though they are incapable of continued survival except in such mixtures. In this respect they differ from the generally reported behaviour of mixtures of mutant and wild type nuclei in heterokaryotic association. It is suggested, however, that the behaviour of either may be atypical, since most of the heterokaryons investigated will have been chosen for their stability, while a cytoplasmic mutant which was not suppressive is unlikely to be recognized and isolated by present techniques.

The phenotypic changes which occur in ageing clones of *Aspergillus glaucus* such as the decrease in rate of growth, pigmentation and perithecial production, have been explained in terms of reversible cytoplasmic changes resulting from readjustments in the equilibrium of normal cytoplasmic constituents (Jinks, 1954, 1956, 1957, 1958; Mather & Jinks, 1958). Sooner or later, however, in every ageing clone an irreversible cessation of growth occurs, which has been previously referred to as vegetative death. Its time of onset varies considerably; it has appeared in some lineages after as little as 5 months of vegetative maintenance while in others it has required as long as 2 years of similar maintenance. In the present paper investigations of vegetative death will be described which show that it results from a permanent heritable change in some cytoplasmic element and hence involves a qualitatively different kind of cytoplasmic change from that responsible for the earlier stages of ageing.

METHODS

The media and cultural conditions used were those described in the earlier papers. All the lineages and sub-clones described in this paper have been derived from the three original homokaryon clones 1, 2 and 4 of *Aspergillus glaucus* (Jinks, 1956) from which they differ by various introduced major gene mutations.

RESULTS

Description of vegetative death

The onset of vegetative death in a colony is marked by the death of only one or very few neighbouring growing tips. This leaves a gap in the otherwise circular growing front of the colony which widens into a sector as the remainder of the circumference continues to grow. This widening gap, however, is not invaded by the neighbouring, and as yet still growing, hyphal tips as would happen, for example, if a similar gap was made by the mechanical removal of a number of growing tips. On the contrary the cessation of growth spreads laterally from the initial sector to the neighbouring growing tips so that an increasing proportion of the circumference of the colony is involved. Estimates of the extent of vegetative death, measured as the percentage of the circumference which had ceased growing, are given for colonies from two ageing lineages of homokaryon 4 in Table 1. The lateral spread of vegetative death within these colonies is shown by the steady increase in the percentages over the four weekly intervals. Unless large numbers of the unaffected tips are used to initiate new colonies as soon as the first dying sector is observed, the cessation of growth spreads to all the growing tips of the colony and further successful propagation is impossible. At this early stage it is still possible to obtain from these transfers some colonies which do not show further dying sectors. The majority of the transfers, however, give colonies which immediately produce multiple dying sectors and it becomes progressively more difficult to obtain from these sectors tips which are capable of even a limited amount of growth before they die (Pl. 1, figs. 1*a*, 2). This can be seen in Table 2 which summarizes the fate of transfers from a colony of lineage 4_1 (Table 1) when 55 % of its circumference had already ceased growing. The transfers are divided into two sets according to whether they were taken from the vicinity of the dying sector or from the still growing part of the colony. Only one of the 6 transfers taken from the growing sector of the dying colony of lineage 4_1 gave a colony which produced no dying sectors and remained normal for the four weeks it was under observation. The colonies obtained from the remaining 12 transfers (Table 2) produced at least one dying sector and hence

Table 1. *The percentage of the circumference of colonies of two ageing lineages of* Aspergillus glaucus *homokaryon* 4 *which had ceased growing, measured at weekly intervals*

	Lineage	
	4_1	4_2
Week	% of circumference which had ceased growing	
1	25	10
2	45	35
3	85	55
4	95	70

ultimately died. However, by making early transfers from the least affected of these colonies, it was possible to obtain further colonies with dying sectors. Unfortunately, no more normal colonies were obtained by this means, but it kept the dying lineage alive while the analyses described later were performed.

Other symptoms were regularly associated with the cessation of growth in vegetative death. In every case so far examined there was an excretion of an intense brown pigment into the medium in the vicinity of the dying hyphae.

Characteristic microscopic symptoms are also associated with the death of the growing tips. Many tips are swollen to five times their normal diameter and their contents, which are initially dense, gradually disappear or are released onto the surrounding medium. At the same time the contents of older cells, some distance behind the growing tips, may disintegrate and the process spread towards the tips.

Table 2. *The number of dying sectors in 4-week old colonies obtained from hyphal transfers of the dying and still growing sectors of a colony of* Aspergillus glaucus *lineage* 4_1

		No. of colonies showing the following frequency of dying sectors			
Transfers from	Transfers tested (no.)	0	1	2	3 or more
Dying sector	7	0	1	1	5
Growing sector	9	1	3	5	0

Occasionally the cessation of growth is not final and a few tips may resume growth after being apparently dead for as long as 2 weeks. This new growth is sparse and mainly submerged; microscopically it appears normal. These recoveries, however, are short lived unless immediate transfers are made to new plates, where the growth is like that of an aged colony. Each such transfer produces multiple dying sectors within the first 2 weeks and no transfer has survived beyond 3 weeks.

Intra-clonal incidence of vegetative death

While vegetative death has never occurred spontaneously in other than severely aged material, all sister colonies of an aged lineage do not necessarily show vegetative death. Similarly, parallel lineages of the same aged sub-clone often differ in the incidence of vegetative death even when they are apparently equally affected by the other changes resulting from ageing. The most complete record of the incidence of vegetative death was provided by five parallel lineages within an ageing sub-clone of homokaryon 1_5. The lineages were initiated by choosing five colonies at random from the asexual progeny of a single colony. These five colonies and the lineages derived from them were cultured under identical conditions and propagated simultaneously, using the same type of inoculum. At any one time each lineage was represented by at least five sister colonies, and when it was necessary to obtain more detailed information the number was raised to twenty. The lineages were followed for a period of 6 months during which time eight successive propagations were necessary. Although the colonies in the later stages of all five lineages showed the usual symptoms of ageing, in four of the lineages no colony showed vegetative death. In the fifth lineage, the later stages of which are summarized in Fig. 1, the first colony to show vegetative death appeared in the sixth propagation. In the seventh and eighth propagations 2 out of 19 and 6 out of 12 colonies respectively showed vegetative death. Thus the proportion steadily rose even though only hyphae from non-dying colonies were used in the propagation (Fig. 1).

Before the occurrence of vegetative death the fifth lineage was indistinguishable from the other four, even for rate of growth, which is the metric most sensitive to differences in ageing. But subsequent to the appearance of vegetative death in colony A2 in the sixth propagation, the mean rate of growth fell from 99·5 % to 79 % of that of the other lineages. Within the fifth lineage the same relationship held. Thus family D (Fig. 1) which alone showed no vegetative death had a significantly higher rate of growth ($P = 0{\cdot}02$–$0{\cdot}01$) than the other colonies of the lineage even after omitting from the latter all colonies which were showing vegetative death at the time of scoring, namely, A2, A31, A32, A33, A34, B4, C11, C12 and E2. Thus a decrease in rate of growth was associated with vegetative death in the fifth lineage and indeed this decrease preceded the morphological symptoms.

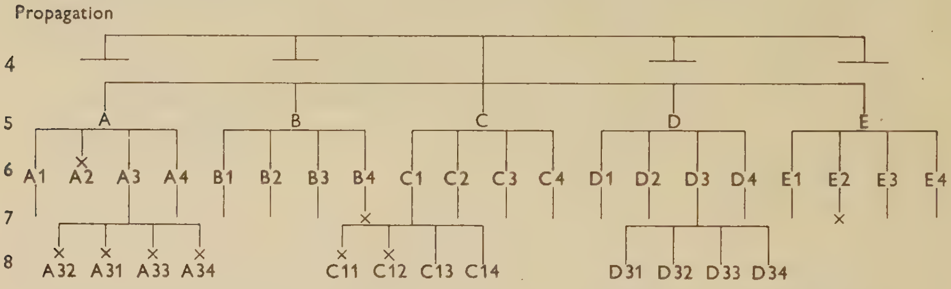

Fig. 1. The fifth lineage of an ageing subclone of *Aspergillus glaucus* homokaryon 1_5 from the 4th to 8th propagation. Colonies showing vegetative death are marked with a ×.

More important, however, is the variation for these associated characters among colonies which have only recently been separated by vegetative propagations, and all of which originated from a single colony in the fourth propagation. From this it follows that either every case of vegetative death is an independent occurrence of the causal change or the causal agent must segregate among vegetative inocula taken from single colonies. Evidence for segregation is presented in the next and subsequent sections.

Method of propagation and intraclonal incidence

In so far as vegetative death has only been encountered in aged material, and indeed appeared to be an end product of the ageing process, all factors which affect the rate of ageing are prospectively important in determining the frequency of vegetative death. Foremost among these factors is the type of inoculum used for propagation (Jinks, 1956). Results presented in the earlier paper amply illustrate these points; they do not, however, include investigations of the effect of method of propagation on colonies which are already showing vegetative death.

Since sexual reproduction is lost quite early in an ageing lineage it is only possible to compare the vegetative and asexual transfers of a colony which shows vegetative death. The transfers made from the asexual spores are initially without dying sectors and it may be some time before they appear. But since asexual spores are not produced in the immediate vicinity of the dying sectors those contained in a sample from a dying colony are obtained preponderantly from the more normal parts. Even so, very few asexual spores germinate, and attempts to improve their viability by the addition of casein hydrolysate (Jinks, 1958) failed. Still fewer asexual spores survive the early stages of growth and give rise to macroscopic colonies. While, however, the proportion of spores which germinate and give rise to macroscopic colonies is always low, the actual proportion varies with the extent of vegetative death in the parent colony. For example, between 2 and 7 % of the spores in random samples from dying colonies of homokaryon 1_5 germinated and from 0·1 to 1·0 %, respectively, gave macroscopic colonies. The latter were superior to their parent colony and 97 % were without dying sectors after 3 weeks of incubation. Their appearance and rate of growth, however, was still that of colonies of an aged lineage, so that their superiority was prospectively short lived.

Vegetative transfers from the still growing portions of the same colonies of homokaryon 1_5 showed between 70 and 100 % death in the first 2 weeks of growth. This performance is inferior to that of the colonies obtained from the asexual spore transfers. But it must be remembered that the spores which give rise to a colony are a highly selected sample of those produced by the dying colony. And presumably if a selection pressure between 1/100 and 1/1000 were practicable with vegetative transfers we might expect to obtain a similar proportion of normal colonies.

Further comparisons between vegetative and asexual propagations of single colonies showing vegetative death were made as follows. Hyphal tips of a dying colony which had ceased growth and which were incapable of further growth when transferred were traced back in the colony to the last head of asexual spores which they produced. Samples of single spores were taken from heads and attempts were made to germinate them and obtain macroscopic colonies. In a dying colony of homokaryon 4, 24 such heads were found which from their proximity to the dead hyphal tips must have been produced simultaneously with the cessation of growth. The results obtained are summarized in Table 3. In all, there were 7 viable spores from 4 heads; of these, 4 spores from 3 of the heads gave macroscopic colonies. And while all 4 colonies had the performance expected in an aged lineage only one showed vegetative death during the three weeks they were observed.

These results are virtually identical with those obtained in the previous experiment. But in addition they show segregation within single heads of spores, each head having originated from a single cell. For example, of 50 spores taken from one of the 24 heads in Table 3, 47 were inviable, 2 gave colonies which soon showed vegetative death and one gave a normal macroscopic colony. Thus cell lineages which undoubtedly contain the causal agent of vegetative death can produce spores, albeit with a low frequency, which are apparently free from the agent or contain it at a much decreased concentration.

Table 3. *The distribution of viable spores, and of spores capable of giving a macroscopic colony, in* 24 *heads from a dying colony of homokaryon* 4.

Approximately 50 spores were taken from each head.

No. of heads sampled	No. of viable spores/sample	No. of macroscopic colonies produced
20	0	0
2	1	1
1	2	2
1	3	1
Totals: 24	7	4

The cytoplasmic basis of vegetative death

Heterokaryon tests. It was previously shown (Jinks, 1954, 1956) that an aged lineage whose colonies as yet showed no signs of vegetative death could be rejuvenated by heterokaryotic exchange of cytoplasm with a vigorous colony. As a consequence colonies of the rejuvenated lineage were still growing normally when those from the original aged lineage were showing vegetative death. At the stage when visible signs of vegetative death appear in the colonies the above situation no longer holds. And in every one of the numerous heterokaryotic combinations which have been made between dying and normal colonies the heterokaryon has behaved like the dying component, producing all the characteristic symptoms of vegetative death shortly after formation. (Pl. 1, figs. 1*a*, 1*b*).

The details of the analysis of these heterokaryons have varied with the available natural and introduced markers in the homokaryons in which vegetative death has occurred. But in all cases the criteria on which the nature of vegetative death has been decided have been the same as those summarized by Jinks (1958); the more important of these emerge in the following examples.

Lineage 4_1 in which vegetative death occurred carried a white asexual spore colour mutation. A heterokaryon was made between a dying colony of this lineage and a vigorous colony of lineage 4_2 which carried a buff asexual spore colour mutation. The asexual spores produced by the heterokaryon were sampled within 7 days of its formation, which was 2 days after it showed vegetative death. Less than 1 % of the spores in random samples germinated and these gave rise to homokaryon colonies with either white or buff spores and heterokaryon colonies with green, white and buff spores in the proportions 1:1·9:0·9, respectively. And although only the white-spored component originally showed vegetative death, heterokaryon colonies and homokaryon colonies with buff spores were obtained which gave all the typical symptoms.

In a further analysis single heads of the buff and white homokaryotic spores produced by the heterokaryon were individually sampled. The results are given in Table 4. The total number of spores taken from each type of head was estimated by counting a small sample, while the number of viable spores and their subsequent performance was exactly determined. It is clear from Table 4 that irrespective of whether the genotype of the spores was that of the dying or vigorous component of the heterokaryon their performance was the same and like that of a dying colony. Furthermore, these samples gave colonies, with the genotype of the initially vigorous component, which showed vegetative death after up to 7 days of normal growth. These results provide strong evidence for the cytoplasmic origin of vegetative death in homokaryon 4; further proof for this particular case is given later.

Table 4. *The performance of asexual spores taken from single heads of buff and white homokaryotic spores produced by the heterokaryon*

	Performance		
	Non-viable	Viable	Showed vegetative death
Sum of 12 heads of white spores:	$\simeq 600$	2	0
Sum of 11 heads of buff spores:	$\simeq 600$	5	3

In other instances no appropriate colour marker was present in the lineage affected by vegetative death. Such a case arose in a lineage of homokaryon 1_5 (Fig. 1) which had the wild type pigmentation. Derivatives of this homokaryon, however, readily form heterokaryons with homokaryon 2 (Pl. 1, figs. 1 *a*, *b*) and both the homokaryons and the heterokaryons can be classified on the microscopic appearance of one or a few hyphae. As adult colonies they are readily distinguishable by colonial morphology, and spore colour differences are superfluous.

The viability of random samples of the asexual spores of the dying component of the heterokaryon and that of the asexual spores produced by the heterokaryon itself, were both between 4 and 5 %. Without autonomous spore colour differences between the components of the heterokaryon, however, it is not possible to decide whether they are equally non-viable, though with such a low mean viability it is unlikely that any of the three types of spores produced by the heterokaryon have a normal viability. The viable fraction gave colonies of homokaryon 1_5 and 2 both of which showed vegetative death with similar frequencies. The time of onset of vegetative death, however, was a little later in homokaryon 2 and the mean diameter of the colonies at death somewhat larger; this is shown in Table 5. Since some colonies die before measurements can be taken, and others are still growing normally some time after their recovery from the heterokaryon, the results in Table 5 have been arbitrarily limited to those colonies which died between 3 and 24 days after germination. This difference in detail in the performance of colonies of homokaryons 1_5 and 2 recovered from the heterokaryon, does not detract from the proof of the cytoplasmic origin of vegetative death. Its origin presumably lies in the genotypic differences between the homokaryons, a subject considered later.

In all, four examples of vegetative death analysed by the heterokaryon test proved to be cytoplasmic in origin. These include examples in homokaryons 1_2 and 2_6 in addition to those just described.

Table 5. *The mean age and mean diameter of colonies of* Aspergillus glaucus *homokaryons* 1_5 *and* 2, *recovered from the heterokaryon, at the time of death.*

	No. scored	Mean age at onset of death (days)	Mean diameter (mm.)
Homokaryon 1_5	36	8·8	15·4
Homokaryon 2	22	10·0	28·6

Spread of vegetative death within colonies

A characteristic feature of a dying colony is the lateral spread of the cessation of growth to neighbouring growing hyphal tips (Table 1). Estimates of the speed of this spread in homokaryon 4_1 showed that the percentage of the circumference of the colonies which had ceased growth increased at the rate of 2·8 ± 0·3 % per day. When inocula containing dying hyphae are placed alongside normal hyphae of the same or of a different colony of the same lineage, in about 70 % of cases the normal hyphae show visible symptoms of vegetative death within 3 to 5 days. Furthermore, the site of the cessation of growth is not random but spreads outwards from the dying inoculum. In this way vegetative death can be induced in vigorous colonies of an unaged lineage of the same clone. For example it has been induced in colonies of the unaged lineage 4_{44} (which has buff spores) on nine separate occasions by placing inocula from the aged lineage 4_1 (which has white spores) in their path. By varying the condition of the dying inocula used in these experiments it has been shown that hyphae which are affected by vegetative death but still capable of some growth must be present for successful induction. It would seem, therefore, that the appearance of vegetative death among neighbouring normal hyphae is the result of an infective spread from the affected hyphae.

In the induction of vegetative death in lineage 4_{44} by lineage 4_1 the production of green heterokaryotic spores consistently preceded the cessation of growth in the neighbouring hyphae of 4_{44}. Furthermore, all the hyphae of 4_{44} which died were formed after these spores were initiated. Also none of the numerous transfers of hyphae and spores produced by lineage 4_{44} after their contact with lineage 4_1, but before the heterokaryotic spores were initiated, gave colonies which showed vegetative death. Yet similar transfers made at a later stage from the affected portions of 4_{44} showed all the typical symptoms.

Such results leave no doubt that the infective spread of vegetative death within colonies is *via* anastomoses between affected and unaffected growing tips, with the ultimate death of the cell lineages which develop from the tips which receive the resulting mixed cytoplasm. Sharpe (1958) used the demonstration of infective spread as an alternative to the heterokaryon test for detecting cytoplasmic differences between two phenotypes; the criteria being the same in both tests. Since in the case of vegetative death the two methods have given identical results further details will not be given.

Reversal of vegetative death

Heterokaryons between dying and vigorous lineages of the same or different clones die soon after formation, but they do live somewhat longer, on the average, than their dying component in the homokaryotic state. Some temporary improvement is, therefore, introduced along with the cytoplasm of the vigorous component. This improvement can be maintained by continuously forming heterokaryons by using hyphae from a vigorous colony. In practice, the heterokaryon between dying lineage 4_1 and vigorous lineage 4_{44} was surrounded by a dense ring of germinating asexual spores from 4_{44}. Heterokaryotic hyphae, recognized by their production of green spores, were isolated as soon as they appeared at the edge of this mass, and transferred to fresh plates. The procedure was then repeated on the longer-surviving transfers. At the third attempt two heterokaryotic colonies were obtained which produced no dying sectors during the 3 weeks they were under observation. The viability of their asexual spores was as high as 42 % and almost all gave adult colonies, irrespective of whether they had the white spores of lineage 4_1 or the buff spores of lineage 4_{44}. Three weeks after germination no homokaryon colony showed vegetative death and the re-extracted homokaryon colonies of 4_1 had a mean rate of growth of 1·53 mm./day compared with the last recorded rate before heterokaryosis of 0·91 mm./day. Thus by this technique colonies of lineage 4_1 were regularly recovered in a relatively unaged condition. The introduction of cytoplasm from the vigorous lineage is the essential part of this improvement but heavy selection for the more vigorous heterokaryotic hyphae is an inevitable result of the technique. However, controls involving the selection of the fastest growing tips of heterokaryons failed to produce colonies which did not show vegetative death.

The nuclear component of vegetative death

During the investigation of vegetative death some instances of nuclear participation emerged. In the first place the frequency of vegetative death varied significantly between identically maintained lineages of different genotypes. This had already been shown by the published frequencies of vegetative death for homokaryons 1, 3 and 6 (Jinks, 1956). It is, however, difficult to judge the importance of these observations in view of the magnitude of the variation in frequency of vegetative death between identically maintained lineages of the same genotype. A clearer example of nuclear participation was given earlier in the present paper where it was shown that the time and stage of onset of vegetative death differed for the two components extracted from a dying heterokaryon.

The remaining example, now to be described, belongs to a different category of nuclear participation. It arose from attempts to induce vegetative death in unaged material by ultraviolet (u.v.) irradiation. Three examples were recovered following the irradiation of homokaryon 2_6 which showed all the visible symptoms of vegetative death (Pl. 1, fig. 3). In the one example analysed in detail, the appropriate tests failed to demonstrate a cytoplasmic component, and unlike our earlier examples of vegetative death, adding casein hydrolysate to the medium raised the viability of its asexual spores to normal. The subsequent behaviour of the colonies obtained from spores plated on the minimal medium and on minimal medium + casein hydrolysate when transferred to the minimal medium is shown in Table 6.

Table 6. *The behaviour of colonies of* Aspergillus glaucus *obtained from spores germinated on minimal medium or minimal medium + casein hydrolysate, when transferred to minimal medium*

Colonies from	Colonies tested (no.)	Performance of colonies on minimal medium		
		Normal	Dying sector	No growth
Minimal medium	26	21	5	0
Casein hydrolysate medium	20	8	4	8

The colonies with a normal phenotype and those which required casein hydrolysate for growth were true breeding, while those which produced dying sectors repeated the segregation of their parent colony (Table 6). Furthermore, the heterokaryon tests revealed only a nuclear difference between the two true-breeding segregants. It would seem, therefore, that the segregating colonies which show vegetative death must themselves be heterokaryons between the wild type segregant and its auxotrophic mutant. From this it follows that the vegetative death resulted from a breakdown of the heterokaryon to give homokaryotic sectors of the auxotrophic mutant which would have been incapable of prolonged growth on the minimal medium when no longer in close cytoplasmic contact with nuclei of the wild type component. This interpretation is supported by the increase up to 100 % in the proportion of spores with the genotype of the auxotrophic mutant, in the vicinity of the growing tips which have ceased growing.

DISCUSSION

These investigations have shown that spontaneous vegetative death in ageing lineages of *Aspergillus glaucus* is cytoplasmic in origin. But the properties of the cytoplasmic change which initiates vegetative death are unlike those responsible for the degeneration in sexual potential, rate of growth, etc. in ageing lineages. The latter involve no irreversible change in the structure or function of cytoplasmic components and are explicable solely in terms of readjustments in the balance of normal cytoplasmic constituents. (Jinks, 1956, 1957, 1958). In contrast, the segregation from mixed cytoplasms of the cytoplasmic change responsible for vegetative death and its infective spread, both in the heterokaryon test and in its various modifications, clearly defines it as mutational in origin (Arlett, 1957; Jinks, 1958; Sharpe, 1958). Thus although ageing lineages regularly end in vegetative death the cytoplasmic change which produces the latter is not, apparently, a continuation of that responsible for ageing. On the other hand, it is difficult not to see a causal connexion between the two, whereby the degeneration of the cytoplasm, resulting from ageing, and the disorganization to which this presumably leads, increases its susceptibility to further breakdown.

It is also clear from the phenotypes of the colonies in the heterokaryon and related tests that in mixed cytoplasms the mutant responsible for vegetative death is suppressive in the sense defined by Darlington (1944). Thus the wild type cytoplasm is suppressed by the action or by the competitive reproduction of the cytoplasmic mutant. Suppression of one component of a mixed cytoplasm by the other appears to be the rule rather than the exception in the examples so far described (Ephrussi, Hottinguer & Roman, 1955; Pittenger, 1956; Arlett, 1957). Occasionally it has been the wild type component which has suppressed the mutant, but more often it has been the opposite. The present examples are particularly remarkable in that the suppressor is incapable of survival except when in mixtures with its wild type counterpart. As a consequence, dying sectors appear soon after these suppressive, yet lethal, cytoplasmic mutants have been introduced into a wild type cytoplasm either by mutation or by infection *via* hyphal anastomoses.

Suppression in mixed cytoplasms is in marked contrast to the behaviour of mutant and normal nuclear genes when contained in the same nucleus. Here the chromosome mechanism ensures that they are kept in step in reproduction and one cannot suppress the other because reproductively they do not compete. Competition at the cytoplasmic level presumably reflects the absence of comparable reproductive controls. A closer comparison might be expected with the behaviour of mutant and normal genes contained in different nuclei within a heterokaryon hypha; under these conditions the mutant and normal nuclei are free to compete in reproduction. In the majority of reported cases they establish equilibrium frequencies which are compatible with optimum growth (Beadle & Coonradt, 1944; Pontecorvo, 1946; Jinks, 1952; Rees & Jinks, 1952; Pontecorvo, 1953). Where optimal growth is obtained only in those hyphae where one component nucleus is in the homokaryotic state the heterokaryotic mixture breaks down. But it does so in favour of its competitively superior component. It is on this last point that the comparison between heterokaryotic associations and cytoplasmic mixtures has most frequently failed, in so far as the competitively superior component of the cytoplasmic mixtures has often been the poorer or even lethal phenotype in the pure state. This contrast, however, may be misleading. Few unstable heterokaryotic mixtures have been examined, if only because stability is a prerequisite for the investigation in which they have been used. It was partly for this reason that the example of vegetative death which was produced by an induced nuclear mutation was given in detail. This example shows that nuclear mutations can be obtained which suppress their partners in heterokaryotic associations even on media where the suppressor is lethal in the homokaryotic state.

On the other side, the behaviour of the relatively few cytoplasmic mixtures described so far may well be atypical. And indeed it is difficult to see how the majority of cytoplasmic mutations would have been recovered if they had not suppressed their normal equivalents in the same or neighbouring hyphae. It seems probable therefore, that as methods of detecting and recovering cytoplasmic mutations improve, and more examples of unstable heterokaryons are analysed, the similarity between heterokaryotic associations and cytoplasmic mixtures will emerge as much greater than it appears at present.

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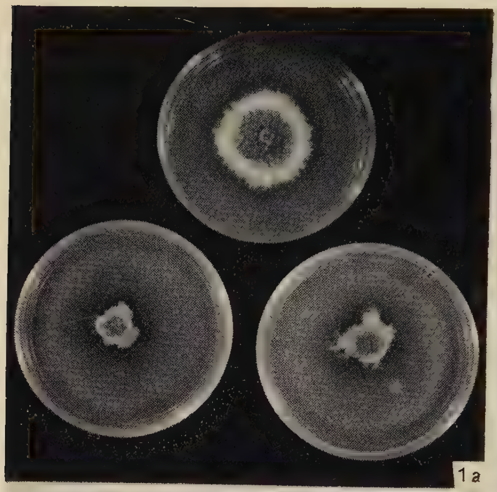
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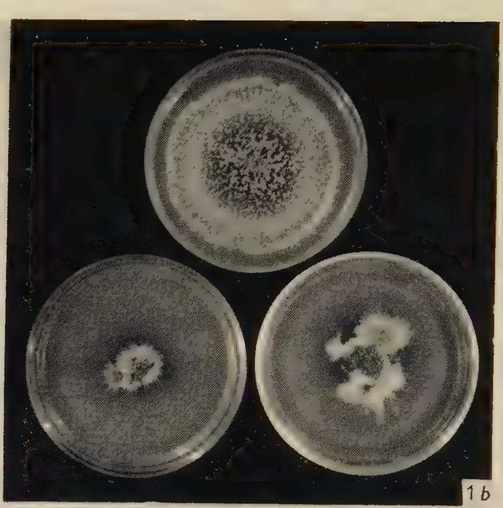
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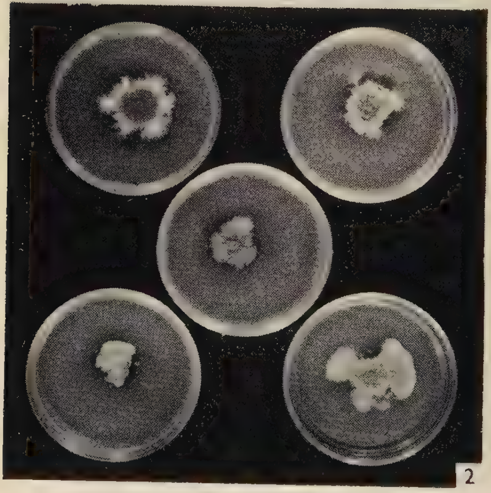
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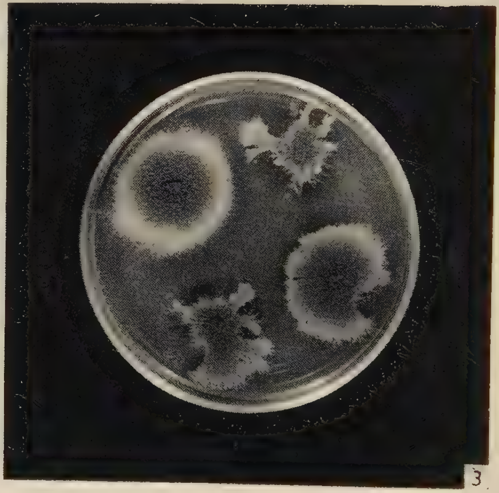
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J. L. JINKS—VEGETATIVE DEATH. PLATE 1

(*Facing p.* 409)

EXPLANATION OF PLATE 1

Fig. 1*a*. Three colonies of the aged fifth lineage of homokaryon 1_5. The bottom two are showing the last stages of vegetative death.

Fig. 1*b*. Three heterokaryons between the aged colonies of homokaryon 1_5 shown in Fig. 1*a* and a vigorous colony of homokaryon 2_6. The phenotypes of the bottom two heterokaryons show the suppressive effect of the lethal cytoplasmic mutation responsible for vegetative death.

Fig. 2. Examples of vegetative death in five different homokaryon clones. Three of the examples, i.e. the two on the left and the centre colony, arose spontaneously in aged lineages. The other two, on the right, were produced in vigorous lineages following heterokaryotic association with a colony showing vegetative death.

Fig. 3. Four sister colonies, each obtained from single asexual spores of a colony which showed vegetative death after u.v.-irradiation. In this example the cause of vegetative death is an induced nuclear mutation requiring casein hydrolysate for normal growth and the symptoms are only shown on the minimal medium.

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A Survey of the Antibiotic Powers of Yeasts

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SUMMARY: Examination of the antibiotic powers towards bacteria and moulds of 150 strains of yeasts from the British National Collection of Yeast Cultures has revealed that whereas none exhibited marked antibacterial properties certain of them inhibited the growth of moulds. Strains of *Candida pulcherrima*, when grown on a malt+yeast+peptone+glucose agar medium inhibited the growth of strains of certain *Mucor*, *Fusarium* and *Penicillium* spp. The inhibition was due to pulcherriminic acid, a derivative of the red pigment, pulcherrimin, formed by *Candida pulcherrima*. It inhibited growth of the moulds completely at 5000 p.p.m. and partially at 500 p.p.m.

Only one systematic study has been reported in which the inhibitory powers, against bacteria and other organisms, of a large number of different strains of yeasts have been observed. In a brief report, Koch (1952) described the screening, by means of a diffusion plate method, of about 150 yeasts including distilling, baking, brewing and other strains against the bacteria, *Bacillus subtilis*, *Serratia marcescens* and strains of Pedicoccus isolated from beer. The yeasts *Brettanomyces bruxellensis*, *Schizosaccharomyces pombé* and brewing strains of *Saccharomyces cerevisiae* and *S. carlsbergensis* were found to inhibit the growth of *B. subtilis* and the pediococci but other species of yeast were found to produce substances which stimulated the growth of the bacteria. No attempt was made by Koch to isolate the substances producing inhibition of bacterial growth. Other workers have used only two or three strains of yeast but have carried out fractionations of the yeast cell constituents and culture fluids to concentrate the active materials. In a review of investigations made before 1949, Florey *et al.* (1949) noted that unsaturated fatty acids from brewers' and bakers' yeasts and from *Debaryomyces mucosus* and *Torulopsis utilis*, and succinic acid or other acidic substances from *Torulopsis utilis* var. *major* possess antibacterial properties. Later work has shown that cyclic peptides from bakers' yeast (Motzel & Cook, 1958), certain proteins from brewers' and bakers' yeast (Parfentjev, 1955, 1957*a*, *b*), the crystalline carotenoid lusomycin from *Rhodotorula glutinis* var. *basitarica* (Prista, 1952) and unidentified substances from *S. cerevisiae* (Skorodumowa, 1954) all inhibit the growth of certain strains of bacteria.

The National Collection of Yeast Cultures provides a very wide selection of yeasts and the opportunity has been taken to survey the antibiotic properties of a representative number of these cultures. At least one strain of each species listed in the catalogue has been tested, by a diffusion plate method, for inhibitory action towards six selected bacteria and against certain moulds. In addition the effects of a number of the concentrated culture fluids from the yeasts on the growth of bacteria and moulds have been observed.

METHODS

Organisms. 153 strains of yeast, comprising Brettanomyces, (5) Candida (31), Cryptococcus (5), Debaryomyces (4), Endomycopsis (3), Hansenula (7), Kloeckera (5), Nadsonia (2), Oospora (2), Pichia (6), Rhodotorula (10), Saccharomyces (44), Schizosaccharomyces (2), Sporobolomyces (5), Torulopsis (13), Trichosporon (3), and one each of Bullera, Hanseniaspora, Lipomyces, Saccharomycodes, Schwanniomyces and Trigonopsis were tested. Some of the bacteria were obtained from the National Collection of Type Cultures, (NCTC) Colindale, London, and the rest had been isolated in the Foundation. The cultures of moulds were kindly supplied by the Commonwealth Mycological Institute (CMI), Kew, England. Details are given in Table 1.

Table 1. *Bacteria and moulds used as test organisms*

	Gram reaction	Strain No.	Growth medium used	Temperature of incubation (°)
(*a*) Bacteria				
Staphylococcus aureus	+	NCTC 6571	Nutrient broth agar	37
Escherichia coli	–	*	Nutrient broth agar	25
Acetobacter sp.	–	*	Acetobacter medium	25
Bacillus cereus	+	*	Nutrient broth agar	25
Flavobacterium proteus	–	*	Deep-liver medium	25
Pseudomonas aeruginosa	–	NCTC 6570	Nutrient broth agar	37
(*b*) Fungi				
Rhizopus stolonifer		CMI 17313	MYPG†	25
Fusarium oxysporum		CMI 53097	MYPG†	25
F. equiseti		CMI 49888	MYPG†	25
Penicillium periculosum		CMI 61383	MYPG†	25
Mucor plumbeus		CMI 74386	MYPG†	25

* These cultures were isolated in the Foundation.
† Malt + yeast + peptone + glucose agar.

Media. The bacteria and moulds were maintained and tested on the appropriate media listed in Table 1. Nutrient broth agar was prepared as described by Mackie & McCartney (1949) and the Acetobacter, deep-liver and malt + yeast + peptone + glucose + agar (MYPG agar) media as described by Haynes, Wickerham & Hesseltine (1955). The yeasts were grown on solid or liquid MYPG medium. The iron-rich medium for cultivation of *Candida pulcherrima* was prepared as described by Cook & Slater (1954).

Materials tested. Pulcherriminic acid (3:6 di-*iso*butyl-4:5-dihydro-2:4-dihydroxy-5-oxo-pyrazine-1-oxide) was isolated as described by Cook & Slater (1956). Culture fluids (500 ml.) from the yeasts *Brettanomyces bruxellensis* (NCYC 362), *Candida pulcherrima* (166, 371, 372, 373), *Candida utilis*

(168, 359), *Saccharomyces carlsbergensis* (112, 450), *Saccharomyces cerevisiae* (232, 240, 447), *Schizosaccharomyces pombé* (132), after 1 week's growth were separated from the organisms by centrifugation at 10,000 rev./min. and freeze-dried. The resulting solid was redissolved in sterile water (5 ml.).

Methods of testing. Three methods were used for detecting growth inhibition.

1. Blocks of agar (1 cm.^{2} and of the thickness of the medium) bearing a single yeast colony were cut with a sterile scalpel from 48 hr. cultures on MYPG agar and placed on the surface of a dry plate of suitable medium previously surface-inoculated with a saline suspension (0·1 ml.) of the bacterium or mould under test. The medium was examined for zones of inhibition of growth around the blocks after aerobic incubation for 1–4 days at 25° or 37° (see Table 1).

2. The sensitivity of certain moulds was tested by streaking a loopful of a young yeast culture directly on to the surface of the appropriate medium previously inoculated with the mould as described above. The plates were then incubated and examined for zones of inhibition as previously described.

3*a*. Freeze-dried culture fluids redissolved in one hundredth of their original volume (see above, 1·66 ml.) were added to molten medium (15 ml.) just before pouring plates. These were then surface-inoculated and incubated and the amount of growth compared visually with that obtained on the control medium devoid of culture fluid.

3*b*. The inhibitory properties of pulcherriminic acid were tested similarly, concentrated solutions in 50 % aqueous pyridine being used to give final concentrations in the medium of 5000, 500 and 50 p.p.m. For comparison, plates were prepared of medium containing the same amount of pyridine but no pulcherriminic acid.

RESULTS

Bacteria. In none of the tests in which bacteria were used as test organisms was their growth inhibited even slightly by substances secreted by the yeasts. In many cases the bacteria showed enhanced growth around the agar blocks containing the yeasts. Neither concentrated culture fluids from some of the yeasts nor pulcherriminic acid produced inhibition of growth.

Fungi. Most of the substances secreted by yeasts appeared also to enhance the growth of the moulds with the one exception, however, that strains of *Candida pulcherrima*, when grown in the presence of Fusarium, Penicillium and Mucor organisms produced zones of inhibition varying from 0·1 to 0·5 cm. diam. around the yeast colony. The size of the zone in which growth of the test organism was prevented varied considerably from one batch of medium to another. The inhibition appeared to be inversely related to the amount of the red pigment, pulcherrimin, formed in the medium. Indeed, when an iron-rich medium (see above) was used and there was a very large production of pulcherrimin by the yeast no inhibition of mould growth was observed. After prolonged growth, the moulds completely overlapped the yeast colony on the latter medium, but did not do so on the normal medium where inhibition of growth was observed. Concentrations of 5000 p.p.m. pulcherriminic acid completely inhibited growth of *Penicillium*, *Fusarium* and *Mucor* spp. but did not inhibit *Rhizopus stolonifer*. Concentrations of 500 p.p.m. of the acid produced a retardation of growth, four days instead of two days being required for full growth. On the other hand culture fluids from *Candida pulcherrima* or from other yeasts produced no significant inhibition of growth. Efforts were made to extract pulcherriminic acid from the freeze-dried culture fluids from *C. pulcherrima* by using pyridine, in which the acid is readily soluble and which was itself without action on the mould. However, only very small quantities of material were obtained which did not inhibit or retard growth of the moulds. All four strains of *C. pulcherrima* from the National Collection (Nos. 166, 371, 372, 373) produced inhibition but strain 373 produced the greatest effect followed by nos. 372, 371, and 166 in descending order of activity. Incidentally, strain no. 373 produced the greatest yield of pulcherrimin on the iron-rich medium.

DISCUSSION

The aim of the present study was to survey rapidly a wide range of yeasts with a view to detecting the production of antibiotic substances of potency comparable to that of the antibiotics at present commercially available; the range of conditions of testing was necessarily restricted. Consequently, substances having weak inhibitory powers or which diffused slowly into the agar, may have gone undetected; this possibly explains the discrepancies between the present results and those of Koch (1952) who reported the presence in brewing and other yeasts of substances which inhibited bacterial growth and brought about lysis of test organisms. Nevertheless, it is clear that the liberal production of antibiotic substances of potencies comparable to those of the commercial antibiotics would have been detected by the methods used. It is concluded that yeasts are not a promising source of antibiotics as compared with certain other organisms.

The inhibition of moulds by pulcherriminic acid is perhaps not unexpected since the compound has been shown to possess structural similarities to the antibiotic aspergillic acid (N-hydroxy-2-oxo-3:6-dibutylpyrazine; White, 1940; Dutcher & Wintersteiner, 1944; Dunn, Gallacher, Newbold & Spring, 1949). From the present results it seems reasonable to presume that in the liquid and iron-rich media most of the pulcherriminic acid which was formed by the yeast was converted, by combination with ferric ion, to pulcherrimin which, being almost completely insoluble in water and organic solvents, did not inhibit growth. In the solid medium, exhaustion of ferric ion in places of yeast growth probably occurred and some pulcherriminic acid was present to produce inhibition of mould growth.

On the basis of the results shown above and of recent findings, a study of the antibiotic properties of other oxo-pyrazines of greater solubility in water than aspergillic acid and pulcherriminic acid may well yield rewarding results.

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The Composition of the Spore Wall and the Wall of Vegetative Cells of *Bacillus subtilis*

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SUMMARY: The spore wall of *Bacillus subtilis* is composed largely of structural protein, in contrast to the peptide + amino sugar + sugar complex of the wall of vegetative cells. Alanine, glutamic acid, $\alpha\epsilon$-diaminopimelic acid, glucosamine, muramic acid, glucose, ribitol compounds and O-ester groups are the principal constituents of the vegetative cell walls. All of the compounds characteristic of the peptide + amino sugar + sugar complex have been detected in the spore wall preparations but they account only for a minor part of the spore wall. The walls of the vegetative bacteria have a high phosphorus content and contain appreciable amounts of a ribitol phosphate polymer. Spore walls and vegetative cell walls differ also in the nature of their N-terminal amino acids; glycine is the principal N-terminal amino acid of the spore wall protein whereas alanine is the main amino acid found in the wall of vegetative cells.

The composition of isolated spore walls or spore 'coats' of several members of the genus *Bacillus* was first investigated by Strange & Dark (1956*a*). Apart from a qualitative study by Yoshida, Izumi, Tani, Tanaka, Takaishi, Hashimoto & Fukui (1957) there have been no reports defining the differences in composition of spore walls and the walls of vegetative cells of spore-forming bacteria. It was apparent from the studies of Strange & Dark (1956*a*) that the spore wall with its high nitrogen content and variety of amino acids would differ from the peptide + amino sugar + sugar complexes encountered in the walls of many Gram-positive bacteria (Cummins & Harris, 1956; Salton, 1953, 1958). The results presented in the present paper summarize some of the chemical differences between the spore wall and cell wall of a strain of *Bacillus subtilis*.

METHODS

Isolation of spore walls and walls of vegetative bacteria. Bacillus subtilis was grown with aeration in nutrient broth at 30°. For the preparation of walls of vegetative bacteria, the organisms were harvested after 16 hr. growth, washed with distilled water and disintegrated by the procedures previously described (Salton & Horne, 1951). After sporulation of *B. subtilis* in the nutrient broth the spores were harvested by centrifugation and washed three times with distilled water. Spores were then crushed by shaking for *c.* 2 hr. with ballotini beads (*c.* 0·13 mm. diameter) on a reciprocating shaker at 0°. The spore walls were washed with distilled water on the centrifuge. Ultraviolet absorption spectra of the spore-wall preparations did not reveal any absorption maxima corresponding to nucleic acids or dipicolinic acid (Powell, 1953) although several very small 'humps' were apparent at wavelengths of *c.* 240 and 280 mμ. Digestion of the spore walls with trypsin eliminated the substances responsible for the ultraviolet absorption. For all experiments reported below, spore walls and cell walls were digested with trypsin (1 mg. trypsin/ml.; 0·1 M-phosphate buffer, pH 8; 2 hr. at 37°) before analysis.

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Chemical analyses

Nitrogen. The nitrogen content was determined on triplicate samples by the procedure described by Johnson (1941) after digestion as in the Kjeldahl method.

Phosphorus was estimated colorimetrically by the Fiske & SubbaRow (1925) procedure.

Amino sugars were determined by the colorimetric method of Rondle & Morgan (1955) after hydrolysis of the wall preparations in sealed ampoules with 2 N-HCl for 2 hr. at 100°. For the individual estimation of glucosamine and muramic acid, the hydrolysates were applied to Whatman paper 3 MM and separation of the basic glucosamine from the neutral muramic acid was achieved by electrophoresis (12 volts/cm., 2 hr., 0·05 M-ammonium acetate buffer, pH 6·3). Muramic acid was eluted from the 'neutral' zone and glucosamine from the 'basic' zone of the electrophoresis paper. Both amino sugars were then estimated by the method of Rondle & Morgan (1955), with glucosamine hydrochloride as the standard. Expressing the muramic acid as glucosamine equivalents underestimates the quantity of this amino sugar in the preparations. Strange & Dark (1956*b*) showed, with crystalline muramic acid, that its colour yield under the conditions of the amino sugar colour reaction is less than that of glucosamine. In the absence of a standard of muramic acid, the glucosamine equivalent at wavelength 530 mμ was multiplied by a factor of 1·78 (Strange & Dark, 1956*b*) to give the muramic acid contents.

Lipid contents were determined by extraction with ether or by ether extraction after hydrolysis of the walls with 6 N-HCl as previously described (Salton, 1953).

O-ester groups were estimated quantitatively by the method of Hestrin (1949); the results are expressed for comparative purposes as O-acetyl contents although the nature of the O-ester groups has not been established.

Diaminopimelic acid (DAP) contents were determined on hydrolysed wall preparations by the colorimetric procedure of Work (1957).

Identification of wall constituents by paper chromatography. Amino acids and amino sugars were identified after two-dimensional paper chromatographic separation (Whatman paper No. 1, pyridine + water, 4:1, v/v; *n*-butanol + glacial acetic acid + water, 6:1:2 v/v) of the 6 N-HCl hydrolysates (16 hr. at 107°). For estimating the relative molecular proportions of the amino acids, the chromatograms were dipped in a solution of ninhydrin (5 %, w/v in acetone + phosphate buffer [9·5; 0·5 by volume]) and the colours were eluted with a mixture of acetone and water (3:1, v/v) and the extinctions determined at wavelength 570 mμ.

Wall constituents possessing free amino groups were identified by reacting the preparations with 1-fluoro-2:4-dinitrobenzene (FDNB) and determining the nature of the dinitrophenyl (DNP) derivatives by paper chromatography and paper electrophoresis of the products liberated by 6 N-HCl hydrolysis as described by Ingram & Salton (1957).

Detection of ribitol compounds. The presence of ribitol phosphate compounds in our cell-wall (but not in the spore-wall) preparations was reported by Baddiley, Buchanan & Carss (1958). With the detection of muramic acid and DAP in the spore-wall preparations it was necessary to examine the spore-wall preparations again, using the Buchanan, Dekker & Long (1950) spray for the detection of polyols. Spore wall (30 mg.) was hydrolysed with 2 N-HCl for 3 hr. at 100°, after removal of the HCl by evaporation *in vacuo* over conc. H_2SO_4 and NaOH pellets, the products were taken up in distilled water and applied to Whatman paper No. 1 as a band (5 cm. wide). The papers were irrigated with *n*-butanol + glacial acetic acid + water (3:1:1, v/v) and the dried papers treated as described by Buchanan *et al.* (1950).

Sugar constituents were identified with the aniline phthalate spray reagent on paper chromatograms of hydrolysates of wall preparations (0·5 N-H_2SO_4, 16 hr. at 100°; followed by neutralization with $Ba(OH)_2$ and de-ionizing with a mixed resin bed of Amberlite IR 120 (H) + IR 4B(OH)). Paper chromatographic separation was achieved on Whatman paper No. 1, using ethyl acetate + pyridine + water (Jermyn & Isherwood, 1949) as the solvent system.

Lysozyme sensitivity was determined as described by Salton (1956).

RESULTS

The results of the quantitative analysis of the spore walls and the walls of vegetative *Bacillus subtilis* are presented in Table 1. The nature of the major cell-wall and spore-wall constituents identified in acid hydrolysates

Table 1. *Analysis of spore walls and walls of vegetative* Bacillus subtilis

	Spore walls	Cell walls
	Dry weight (%)	
Total N	12·9	4·6
Total P	1·4	4·2
Total amino sugars (as glucosamine)	2·2	10·7
Glucosamine	1·5	7·9
Muramic acid	0·6 (1·1)*	2·3 (4·1)*
Diaminopimelic acid	*c.* 1	5·6
Lipid		
a. ether extractable	1·4	0·2
b. total†	3·0	0·7
O-ester groups (as O-acetyl)	0·027	0·255

* Values in brackets represent muramic acid contents corrected by the factor of 1·78 (Strange & Dark, 1956*b*).

† 'Total lipid' estimated by ether extraction after hydrolysis according to Salton (1953).

together with the identification of compounds possessing free amino groups are summarized in Table 2.

Digestion of the walls of vegetative *B. subtilis* with lysozyme liberated 93 % of the weight of the walls as soluble components, leaving behind an insoluble wall residue accounting for 7 % of the original wall material.

DISCUSSION

The results of the analysis of the spore walls of *Bacillus subtilis* reported above are in close agreement with those obtained by Strange & Dark (1956*a*). Although the spore-wall preparations contain the components characteristic of the peptide + amino sugar complex of the wall of vegetative organisms, these constituents represent only a minor part of the spore wall. It seems unlikely that these components arise from contamination with vegetative walls, since glucose, a constituent of the latter, was not detectable in the spore-wall preparations. Strange & Dark (1956*a*) also observed the presence of the 'spore peptides' in the spore-coat fractions of other *Bacillus* species.

Table 2. *A comparison of some of the products identified in acid hydrolysates of walls of spores and of vegetative cells of* Bacillus subtilis

	Spore walls	Cell walls
Amino acids (Relative molecular proportions in brackets)	lysine (6); glycine (5); serine (4); alanine (4); glutamic acid (3); valine/methionine (2); leucine/isoleucine (2); tyrosine (1·5); aspartic acid (1); DAP (1); threonine (1); arginine, cystine, histidine, proline and phenylalanine present in smaller amounts.	alanine (4); glutamic acid (2); DAP (1); traces of aspartic acid, serine, valine and leucine.
Amino sugars	glucosamine, muramic acid	glucosamine, muramic acid
Sugars	none detectable in 30 mg. hydrolysed wall	glucose
Products of reaction with FDNB	DNP-glycine; trace DNP-alanine; mono DNP-DAP (1)*	DNP-alanine; mono DNP-DAP (5)*
Ribitol compounds†	Small amounts detectable in 30 mg. hydrolysed wall	Readily detectable in 5 mg. hydrolysed wall

* Relative proportions of mono DNP-DAP in the preparations.
† Detectable mainly as the anhydro-ribitol compound.

The marked difference between the phosphorus contents of spore walls and of vegetative cell walls can be accounted for by the ribitol phosphate polymer as a major wall component of the vegetative cells (Baddiley *et al.* 1958). However, the spore wall contained 1·4 % P and the results of the paper chromatographic detection of ribitol compounds gave the impression that there was more phosphorus than could be accounted for by the small amounts of the ribitol phosphate polymer found. It is evident therefore that the spore wall contained phosphorus compounds other than the ribitol phosphate polymer (the teichoic acids of Armstrong, Baddiley, Buchanan, Carss & Greenberg,

1958) and lipids. Yoshida *et al.* (1957) observed a small difference between the P-content of the walls of spores and of vegetative cells of *Bacillus cereus*. The presence of O-acetyl groups in bacterial cell walls was first reported by Abrams (1958). Armstrong *et al.* (1958) found alanine as an O-ester group in the techoic acids. We have not determined whether the O-ester groups of the spore and cell-wall preparations are exclusively O-alanyl groups or whether there are also O-acetyl groups.

The amount of lipid that we found in the walls of *Bacillus subtilis* is comparatively low; it agrees with the data of Strange & Dark (1956*a*). However, a lipid content of 3 % could readily account for the hydrophobic properties of bacterial spores if the lipid were oriented on the outer surface of the spore wall.

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Use of Superinfecting Phage for Estimating the Division Rate of Lysogenic Bacteria in Infected Animals

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SUMMARY: The rate of division of lysogenic bacteria in infected animals can be estimated by superinfecting the bacteria with a suitable mutant of the prophage before inoculation. Since, as shown by other workers, the mutant phage is usually stable and does not multiply during bacterial growth, the proportion of bacteria carrying the mutant steadily falls in a predictable manner in each bacterial generation. Comparison of the proportions of superinfected bacteria present at the start and at the end of a known period therefore gives the number of bacterial generations occurring in a known time. It is assumed that there is no selection either for or against superinfected bacteria.

Mice were inoculated with *Escherichia coli* strain K12, lysogenized by phage λb and superinfected by phage λhc. The proportion of superinfected bacteria in the spleen did not usually change in the 30 hr. following inoculation. Hence, this strain of bacteria cannot produce viable progeny in the spleen.

The behaviour of the superinfecting phage differed in two ways from that described by previous workers. First, only part of the adsorbed phage was recovered after induction by ultraviolet radiation. Second, the proportion of bacteria which yielded superinfecting phage sometimes fell very rapidly when superinfected overnight cultures were diluted in a medium which allowed bacterial multiplication. This phenomenon is referred to as 'unstable superinfection'.

When mice are injected with *Salmonella paratyphi* B or *S. typhimurium* and the number of viable bacteria/mouse are counted at intervals thereafter, the viable count is found to increase far more slowly than when the organisms are growing in nutrient broth (Meynell & Meynell, 1958, Table 2). This difference might arise in two ways. Either the bacteria are merely dividing more slowly *in vivo* and the rate of division equals the rate of increase of the viable count; or the bacteria are dividing as fast *in vivo* as in broth but are at the same time being killed, so that the rate of division is greater than the rate of increase of the viable count. The two possibilities can be distinguished only if bacterial division rate can be measured while bacteria are also being killed. This can now be done by the method described below which has been tested with *Escherichia coli* administered to mice by intravenous injection and later recovered from the spleen.

The technique is based on observations made by Bertani (1953, 1954), Jacob & Wollman (1953) and Jacob (1954) on lysogenic bacteria; i.e. bacteria carrying temperate phage in the prophage state (Lwoff, 1953; Bertani, 1958). When a lysogenic bacterium is superinfected with a suitable mutant of its prophage, the mutant enters the bacterium but its subsequent multiplication is usually prevented. Hence, when the superinfected bacterium divides to produce two daughter bacteria (Fig. 1), both contain the original prophage but only one contains the superinfecting phage. Similarly, in the second generation all four daughters carry prophage, but again only one carries superinfecting phage. Thus, if each bacterium initially contains one superinfecting phage particle, the proportion of superinfected bacteria halves in each generation. This will still be so if some bacteria die. For example, if there were 1000 superinfected bacteria initially, there would be 2000 in the first generation of which $\frac{1}{2}$ carried the superinfecting phage. If $\frac{3}{4}$ of the bacteria now died, the fraction of superinfected bacteria would still be $\frac{1}{2}$ (= 250/500).

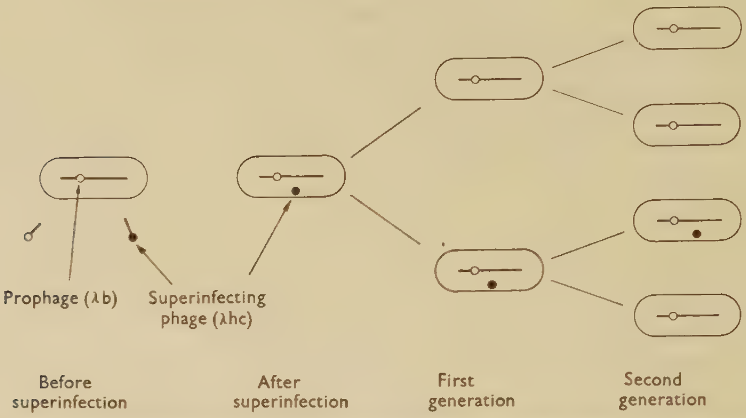

Fig. 1. Distribution of superinfecting phage amongst dividing lysogenic bacteria. Each bacterium carries the prophage of phage λb, which multiplies synchronously with the bacteria. The superinfecting phage (λhc) does not multiply. Hence, if each bacterium initially contains one superinfecting phage particle, the proportion of superinfected bacteria halves in each bacterial generation.

The principle of the method is therefore, first, to inoculate mice with bacteria of which a known proportion is superinfected; and, second, after recovering the organisms from the mouse at a known time after inoculation, to remeasure the proportion of superinfected organisms and deduce the number of generations that have occurred since inoculation. It is assumed that super-infection has no effect on the behaviour of the organism *in vivo* or on its liability to be killed by the host, and also that the fate of the superinfecting phage is not affected by the time taken for bacterial divisions to occur.

The organism used in these experiments was *Escherichia coli* strain K 12, lysogenized by phage λb. This phage forms target plaques which are bigger than those of wild-type phage λ and has the wild-type host range, since it grows in *E. coli* strain C600 but not in *E. coli* strain CR63. Lysogenic bacteria of strain K12 (λb) were superinfected with phage λhc, which forms clear plaques and can grow in strain CR63 (Appleyard, McGregor & Baird, 1956) as well as in strain C600. Strain CR63 is therefore a selective bacterial indicator for the superinfecting phage λhc. Both phage λb and phage λhc are normally latent within the superinfected bacterium and in order to determine the proportion of superinfected organisms the phages must be induced to enter the lytic cycle of multiplication by exposure to ultraviolet (u.v.)

radiation (Lwoff, Siminovitch & Kjeldgaard, 1950). After irradiation the organisms are plated with strain CR63 and with strain C600 before lysis occurs. Each induced bacterium yields phage λb and can form a plaque on strain C600. Each induced superinfected bacterium also yields phage λhc and therefore can form a plaque on strain CR63. The proportion of superinfected organisms is then obtained from the ratio of the number of plaques on strain CR63 to the number of plaques on strain C600.

METHODS

Media. The following solid medium was used for colony and plaque counts by the overlay method (Adams, 1959): peptone 2% (w/v; Evans Medical Supplies Ltd., Ruislip, Middlesex); NaCl 0·5% (w/v); agar 1·5% (w/v) dissolved in distilled water. After adjustment to pH 7·2 by adding N-NaOH, the medium was sterilized by steaming for 2 periods of 45 min. Before plates were poured, glucose was added to a final concentration of 0·2% (w/v). Overlays contained 2·5 ml. of the medium with the agar decreased to 0·6–0·9% (w/v).

Cultures were also grown in buffered glucose peptone water (BGPW) containing: glucose, 0·1% (w/v); peptone 1% (w/v); NaCl 0·5% (w/v); sodium β-glycerophosphate 3·15% (w/v); dissolved in distilled water. The pH was brought to 7·2 by adding N-HCl and the medium finally sterilized by autoclaving at 115° for 10 min.

Buffer. M/20 sodium β-glycerophosphate adjusted to pH 7·2 by addition of N-HCl and sterilized by autoclaving at 115° for 10 min.

Bacteria. Stocks of *Escherichia coli* strain K12 (λb) and the indicator strains, C600 and CR63, were kept on slopes of Dorset's egg medium at 4°. Plates bearing discrete colonies were prepared for daily use and were also kept at 4°. Cultures in liquid medium were grown overnight at 37° in screw-capped bottles containing 10 ml. buffered glucose peptone water which were rotated on a turntable at 45 rev./min. After incubation the cultures had a pH value of 6·7 and contained *c.* 7×10^8 organisms/ml. of which not less than 70% were capable of colony formation. Phage λhc was stable in the supernatant fluids of these cultures.

Phage. Stocks of phage λhc were made by inoculating overlays with strain CR63 and about 10^8 plaque-forming particles of the phage. (Hereafter, the term 'phage' is used instead of 'plaque-forming particle'.) Neither the plates nor the overlays contained glucose. After incubation overnight at 37° the overlays were homogenized in the following medium: buffer, 3 parts; infusion broth, 1 part 1% (w/v) gelatin, 0·01 part. After removal of bacteria and agar by centrifugation and filtration through a sintered glass filter of maximum pore diameter 1·5μ, the titres of the stocks were *c.* 2×10^{10} phage/ml.

Ultraviolet irradiation. The source was a 20 watt discharge tube (Hanovia Ltd., Slough). Before irradiation, organisms in buffered glucose peptone water were diluted 1/100 or more in buffer; organisms recovered from mice were washed as described below. Samples of 5 ml. were u.v.-irradiated at room temperature in Petri dishes placed on a rocker 52 cm. below the tube. Irradiation of phage T2 under these conditions caused 99 % inactivation in 55 sec. After irradiation, organisms were incubated in buffered glucose peptone water for 50 min. at 37° in the dark to prevent reversal of the effect of irradiation (Weigle & Delbrück, 1951). *Escherichia coli* strain K12 (λ), lyses not less than 60 min. after irradiation (Weigle & Delbrück, 1951).

Preparation of superinfected culture for inoculation. Phage λ adsorbs best to *Escherichia coli* strain K12 in the stationary phase of growth (Lieb, 1953). Overnight cultures were therefore used for superinfection. The bacterial concentration was first estimated with a nephelometer (Evans Electroselenium Ltd., Bishop's Stortford, Herts). The culture was then brought to 37° and mixed with the appropriate amount of phage λhc for 15 min. More than 99 % of the phage was absorbed in this time, so that the mean number of phage adsorbed/bacterium could be taken as the initial phage concentration/the initial bacterial concentration. The numbers of viable bacteria and the titre of unadsorbed phage were measured in every experiment.

The superinfected culture was usually chilled to 4°, centrifuged and resuspended in enough cold buffer to give 10^9 bacteria/ml. The suspension was kept in an ice bath while each mouse was given 0·3 ml. of suspension by injection into a tail vein. When the mice had been injected, the remaining suspension was diluted 1/100 in buffered glucose peptone water at 37° and samples irradiated immediately, and 20 and 40 min. later, to determine the proportion of superinfected organisms in the inoculum.

Recovery of the organisms from the spleen and determination of the proportion of superinfected organisms. Mice were killed by cervical dislocation. Measured volumes of blood were taken from the right pleural cavity and added to 25 vol. of buffer to stop clotting. Spleens were ground in 10 ml. distilled water in Griffith's tissue homogenizers. The spleen suspension was next centrifuged in an angle centrifuge for 13 min. at 3000 *g* to separate the organisms from tissue protein which would have absorbed u.v. radiation. The pellets were not compact because of the high density of the original suspension. Most of the supernatant fluid was discarded and the deposit resuspended in buffer and centrifuged again for 5 min. at 3000 *g* to give a compact deposit. This was repeated once and the final deposit suspended in 5 ml. buffer. After u.v.-irradiation for 90 sec. the suspension was added to 1 ml. of 6 times full strength buffered glucose peptone water and incubated as usual before plating. Control experiments showed that the washed suspension contained 70–100 % of the viable organisms present in the spleen before washing, and did not diminish the inactivation of phage T2 by u.v. radiation, showing that all tissue protein had been removed.

Mice. The mice belonged to the Glaxo Strong strain and were bred at St Thomas's Hospital Medical School. Either sex was used, provided the weight was at least 23 g.

RESULTS

Behaviour of superinfected bacteria in vitro

Before the proposed method could be used the optimal conditions for induction by u.v. radiation had to be determined. During these experiments it became clear that the behaviour of phage λhc and *Escherichia coli* strain K12 (λb) differed not only from that of phage λgc, another mutant of phage λ, and the strain of K12 (λ) described by Jacob (1954), but also from that of superinfected strains of *Shigella dysenteriae* and of *Pseudomonas pyocyanea* (*Aeruginosa*) described by Bertani (1954) and by Jacob & Wollman (1953), respectively. The first difference found was that only part of the adsorbed phage was recovered after induction by u.v. radiation. The second difference was that the proportion of bacteria from which superinfecting phage could be recovered sometimes decreased very rapidly when the organisms were diluted into fresh medium after superinfection in the stationary phase, a condition referred to here as 'unstable superinfection'. The following sections therefore deal first with stably superinfected organisms, their response to u.v.-irradiation and their relation to the superinfecting phage, and lastly with unstably superinfected organisms.

Stable superinfection

In most experiments the proportion of bacteria which yielded superinfecting phage after induction did not change during the lag phase. These organisms will be referred to as 'stably superinfected', to distinguish them from superinfected bacteria which inactivated the adsorbed phage during the lag phase.

Induction by ultraviolet irradiation. Stably superinfected bacteria in either the stationary or the logarithmic phase of growth were exposed to u.v.-irradiation for various times and the proportion of induced organisms yielding the superinfecting phage then measured (Fig. 2). The proportion of organisms induced in either phase of growth was greatest with an exposure of 90 sec. (Fig. 2: points joined by continuous lines). However, the proportion of induced bacteria which yielded the superinfecting phage was constant for all exposures.

Fate of adsorbed phage in stable superinfection. The initial proportion of stably superinfected bacteria was always less than expected from the mean number of phage adsorbed/bacterium. This discrepancy might have arisen because (1) all the phage adsorbed by some of the bacteria might be inactivated while all that adsorbed by the remaining bacteria might be preserved; or (2) some bacteria might be unable to adsorb phage so that all the adsorbed phage was taken up by only a fraction of the bacteria; or (3) the bacteria might be identical in their ability to adsorb phage and each phage particle might have a probability (< 1) of being preserved after adsorption. It will be shown below that for each hypothesis, the initial mean number (N) of superinfecting phage preserved/stably superinfected bacterium will be different, so that the hypotheses can be distinguished by estimating N. This was done by allowing a superinfected culture to multiply and determining the relation between the proportion of superinfected organisms and the number of generations of bacterial growth.

Figure 3 shows the result of this experiment and also the curves predicted by the first and third hypotheses given above. All the curves predicted for $N > 1$ have the same general shape, being 'multi-hit' curves of the type '1 hit on each of m sites' (Atwood & Norman, 1949), the number of 'sites' in this system being N. Each curve has a shoulder because an appreciable number of bacteria each contain more than 1 superinfecting phage particle initially so that the proportion of superinfected organisms falls by less than half in each of the first few generations. However, after a number of generations each superinfected bacterium contains only 1 superinfecting phage particle so that the curve becomes linear with slope corresponding to a halving of the proportion of superinfected organisms in each generation. The number of generations required to reach this stage will increase as N increases, so that the larger N becomes, the wider will be the shoulder of the curve. The value of N is obtained from such a curve by extrapolating the linear part to the left until it intersects the ordinate for the end of the lag phase. The point of intersection corresponds to the sum of log N + the logarithm of the initial proportion of stably superinfected bacteria. The reason is that the straight line obtained by extrapolation is that which would have been observed if,

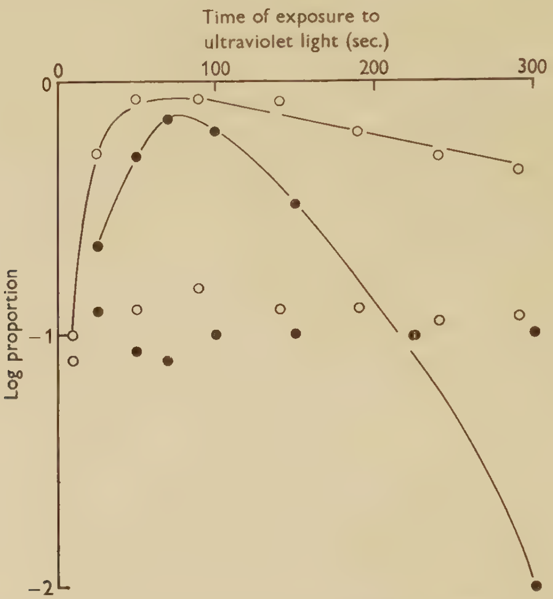

Fig. 2. Effect of time of exposure to ultraviolet radiation on the proportion of organisms induced (points joined by a continuous line) and the proportion of induced organisms yielding superinfecting phage (unjoined points) in either stationary phase (–●–) or logarithmic phase (–○–) cultures. The proportion of induced organisms yielding superinfecting phage is constant, regardless of the proportion of organisms induced.

instead of having a certain initial number of bacteria each containing a mean of N particles, there had been initially N times as many bacteria each containing only 1 superinfecting phage particle. Since the initial proportion of stably superinfected bacteria is known by experiment, the point of intersection with the ordinate provides an estimate of N and enables the three hypotheses given above to be distinguished.

In the experiment shown in Fig. 3, the mean number of phage adsorbed/bacterium (whether superinfected or not) was 2 (log = 0·3) so that the expected proportion of superinfected bacteria was 0·87. However, the observed proportion was 0·36 (log = $\bar{1}$·56). (Logarithms of numbers less than 1 are written with a negative characteristic and a positive mantissa. Thus, $\log_{10}$ of 0·2 is not written as −0·7 but as $\bar{1}$·3, signifying −1+0·3.) If the first hypothesis is valid and each stably superinfected bacterium preserved all the phage it adsorbed, N = mean number of phage adsorbed/bacterium, divided by the proportion of bacteria adsorbing phage = 2/0·87 = 2·3 (log = 0·36). The

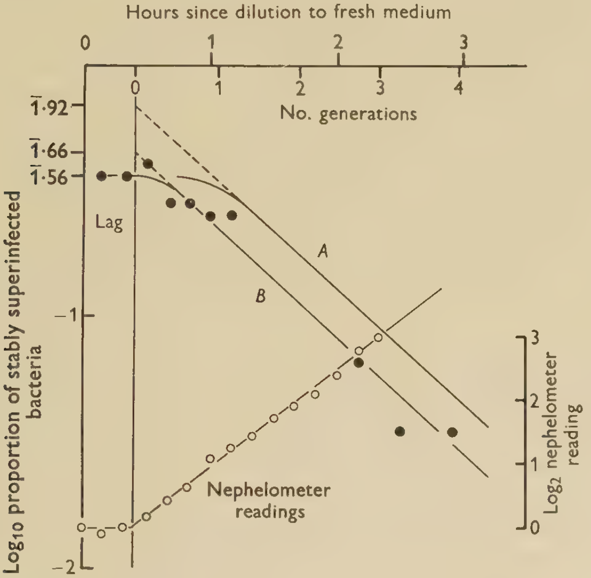

Fig. 3. The proportion of stably superinfected bacteria in the first four generations after superinfection. The degree of bacterial multiplication was measured nephelometrically. The points are plotted at the bottom of the figure against the time scale in hr. on the abscissa and the scale on the right of the figure giving $\log_2$ of the nephelometer readings. The latter scale gave the number of generations which was then plotted on the abscissa against the corresponding time in hr. The mean generation time was 40 min.

Curves *A* and *B* (continuous lines) are the curves predicted by the 1st and 3rd hypotheses discussed in the Text for the relation between the log proportion of stably superinfected bacteria and the number of generations. The points lie on Curve B and not on Curve *A*. The mean number of phage adsorbed per bacterium was 2. The probability per adsorbed particle of being preserved was 0·23.

The dashed lines are produced by extrapolating the descending linear portions of Curves *A* and *B* to the left until they intersect the ordinate for the end of the lag phase. The logarithms of the points of intersection are $\bar{1}$·92 (Curve *A*) and $\bar{1}$·66 (Curve *B*) respectively, as shown on the ordinate for the beginning of the lag phase.

curve predicted on this hypothesis (Curve A in Fig. 3) therefore extrapolates to a point whose logarithm is $\bar{1}{\cdot}92 = 0{\cdot}36 + \bar{1}{\cdot}56$ ($=\log N +$ the logarithm of the initial proportion of stably superinfected bacteria). The observed points clearly do not lie on this line and the first hypothesis is therefore excluded.

The second hypothesis is that 36% of the bacteria adsorbed all the superinfecting phage. The mean number of phage adsorbed/bacterium was 2 so that N now equals $2(1/0{\cdot}36) = 5{\cdot}6$. The predicted curve will be even farther to the right of the observed points in Fig. 3 than Curve A because N is larger (5·6 compared with 2·3). This hypothesis is thus also excluded.

The third hypothesis is that each particle had a constant probability (< 1) of being preserved after adsorption and the expected curve is calculated as follows. On this hypothesis, m, the initial mean number of phage preserved/bacterium (whether stably superinfected or not) is obtained from the initial proportion of stably superinfected bacteria by applying the first term of the Poisson series in the usual way (Adams, 1959); i.e. $0{\cdot}36 = 1-e^{-m}$ giving $m = 0{\cdot}45$. Therefore, N, the initial mean number of phage preserved/*stably superinfected bacterium* $= 0{\cdot}45\,(1/0{\cdot}36) = 1{\cdot}25$ ($\log = 0{\cdot}1$). The predicted curve (curve B in Fig. 3) therefore appears to arise from a point whose logarithm is $\bar{1}{\cdot}66 = 0{\cdot}1 + \bar{1}{\cdot}56$ ($= \log N$ on this hypothesis + log of the initial proportion of stably superinfected bacteria). Curve B in Fig. 3 is clearly in good agreement with the observations.

Hence, it appears that the bacteria were not heterogeneous in their ability to absorb or to destroy superinfecting phage, each particle of which must have had a constant small probability of being preserved after adsorption. The value of this probability in the above experiment was 0·23 ($= 0{\cdot}45/2$ = mean number of phage preserved/mean number of phage adsorbed).

Distribution of superinfecting phage amongst dividing bacteria

Figure 3 showed that the preserved fraction of adsorbed phage is distributed amongst the dividing bacteria for at least 4 generations in the expected manner. Fig. 4 shows the result of an experiment of the same kind in which the bacteria passed through 18 generations. Up to the 10th generation the points agree well with the predicted curve obtained in the same way as Curve B in Fig. 3. After the 10th generation, however, the proportion of superinfected bacteria became constant, indicating that the phage was multiplying synchronously with the bacteria. The explanation almost certainly was that the superinfecting phage lysogenized about 1/1000 of the original bacteria which multiplied like the rest of the bacterial population but were not detected until their number exceeded the number of bacteria carrying non-multiplying superinfecting phage.

Unstable superinfection

Figure 5 shows the changes in viable count and in the proportion of organisms yielding superinfecting phage after induction when an unstably superinfected overnight culture was diluted 1/100 in buffered glucose peptone water at 37° after the 15 min. period allowed for phage adsorption. Half the bacteria died in the first 20 min. but the proportion of organisms which yielded superinfecting phage fell about 25-fold. Fig. 5, Curve B, also shows that the proportion of bacteria which yielded superinfecting phage only fell by half when part of the same culture was centrifuged at the end of the period allowed for phage adsorption, resuspended in buffer at 18° and placed in the refrigerator at 4°. This suggests that the phage disappeared only when the bacteria were placed in a medium which allowed bacterial multiplication. This conclusion was confirmed by the results given in Table 1 which show: (1) that the superinfecting phage was unstable when the bacteria were diluted in fresh medium immediately after superinfection (Sample 1); (2) that the phage did not disappear when the superinfected culture was not diluted (Sample 2); (3) that holding the superinfected culture at 18° for 30 min. before incubation at 37° did not result in stable superinfection (Sample 3).

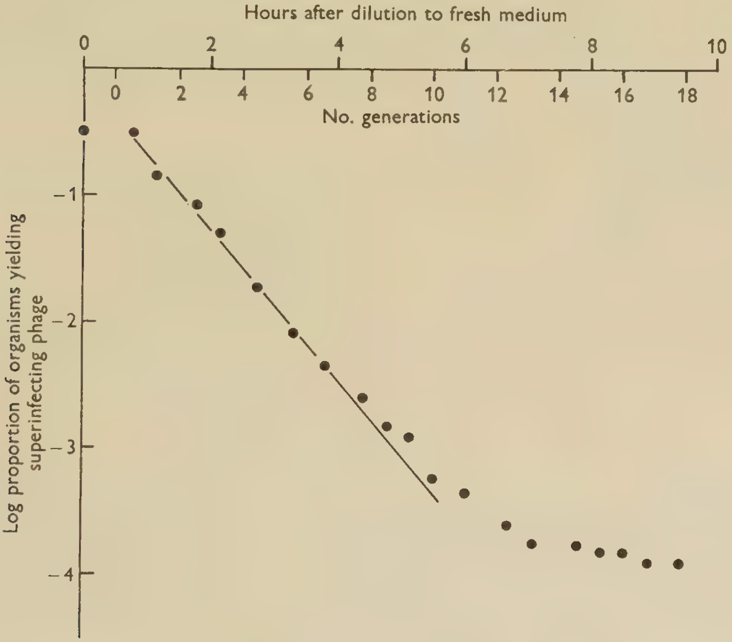

Fig. 4. The proportion of stably superinfected bacteria in the first 18 generations after superinfection. The observations agree well with the predicted curve until the 10th generation. Thereafter the proportion tends to become constant, presumably owing to the multiplication of those bacteria of the superinfected culture which became lysogenized. The mean generation time was 30 min.; the mean number of superinfecting phage adsorbed per bacterium was 3·5; and the probability per adsorbed particle of being preserved was 0·1.

The cause of the instability was not established but was probably associated with the state of the bacteria at the time of superinfection, since the degree of instability varied greatly in different experiments performed under apparently identical conditions. It was established that the degree of instability was the same when the mean number of phage adsorbed was either

4·5 or 0·09, and was decreased when the overnight culture was aerated in buffer + 1 % (w/v) glucose for 100 min. before superinfection. The disappearance of the superinfecting phage was not accompanied by induction of the superinfected bacteria.

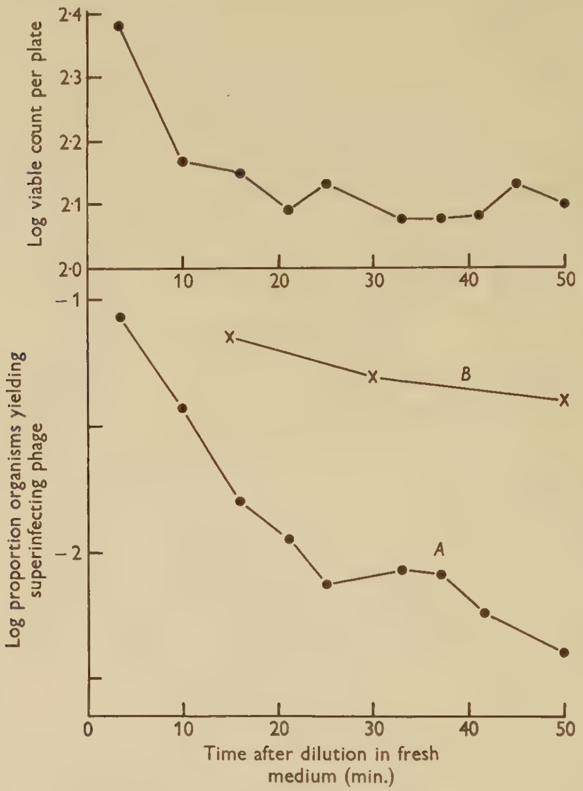

Fig. 5. Behaviour of unstably superinfected organisms during the lag phase. The viable count did not change during the adsorption period of 15 min. The upper curve shows that half the bacteria died after dilution in buffered glucose peptone water at 37° and that the proportion of bacteria yielding superinfecting phage after induction fell 25-fold (Curve A in the lower half of the Figure). Curve B shows that more superinfecting phage was preserved when part of the same culture was transferred to buffer at 18° after superinfection and then placed in the refrigerator.

Behaviour of superinfected bacteria in vivo

Each mouse received about *c.* $2{\cdot}5 \times 10^8$ organisms by intravenous injection and about 10 % of the mice died in the 6 hr. following inoculation.

Fate of organisms after intravenous injection. The viable count in the blood fell very rapidly after inoculation of the organisms; most blood cultures were sterile after 30 min., and when positive never yielded more than 1/1000 of the initial count expected on the assumption that the inoculum was evenly distributed in a blood volume of 5 ml. As blood cultures did not again become positive, the organisms were presumed to be confined to the cells of the reticulo-endothelial system almost from the start of the infection.

The spleen was chosen for these experiments since it contained as many organisms as the liver but, after homogenization, yielded far less tissue protein to be removed before u.v. irradiation. Figure 6 shows that the number of viable organisms recovered from the spleen was rarely more than 1/100 of that inoculated and rapidly decreased in the first 12 hr. after inoculation. After that time, the viable count changed very little, suggesting that the viable organisms recovered in this period might be mutants which were better able to survive in the spleen than the rest of the inoculated population. Therefore, 27 clones, isolated from infected spleens 1 day after inoculation, were tested by re-inoculation into mice. In the first 5 hr. after inoculation these organisms survived no better in the spleen than organisms of the original culture and were, therefore, not mutants of increased resistance.

Table 1. *Behaviour of an unstably superinfected culture under various conditions*

An overnight culture of *Escherichia coli* strain K12 (λb) was superinfected with phage λhc, the mean number of phage adsorbed/bacterium being 1·6. The culture was then diluted in three ways as shown by the second column below.

Sample no.	Treatment after superinfection	Time between dilution and irradiation (min.)	Proportion of organisms yielding superinfecting phage
1	Immediately diluted	0	$1{\cdot}8 \times 10^{-2}$
	1/100 in BGPW* at 37°	30	$1{\cdot}33 \times 10^{-4}$
2	Kept at 37° for 1 hr.	0	$1{\cdot}1 \times 10^{-2}$
	and then diluted 1/100 in BGPW at 37°	30	$1{\cdot}25 \times 10^{-4}$
3	Diluted 1/100 in BGPW	0	$1{\cdot}65 \times 10^{-2}$
	at 18° and after 30 min.	30	$3{\cdot}08 \times 10^{-3}$
	warmed to 37°	50	$5{\cdot}9 \times 10^{-4}$

* BGPW = buffered glucose peptone water.

Changes in the proportion of superinfected bacteria after inoculation

A total of 53 mice were injected with superinfected bacteria in 7 experiments. The results of 4 experiments with 38 mice are given in Tables 2–5 and those from the two of the largest experiments (Tables 2, 3) are also shown in Fig. 7.

The proportion of superinfected organisms in the spleen usually did not change in the 30 hr. following inoculation although it occasionally decreased about 4-fold. This finding suggested that in most mice the viable organisms recovered from the spleen had not divided since the time of inoculation.

Tables 2–5 also show that the proportion of superinfected organisms in the spleen was always greater than in the inoculum, notably in the experiment shown in Table 5; for this reason no attempt was made to calculate the number of generations of growth. There were two possible explanations for this discrepancy. First, the spleen may have preferentially removed superinfected organisms from the blood. Second, some of the inoculated organisms might have been unstably superinfected. There was always a short delay before u.v.-irradiation of the inoculum; this might have allowed these organisms to enter the lag phase and to inactivate some of the superinfecting phage. No inactivation would be expected to occur after the organisms had been injected

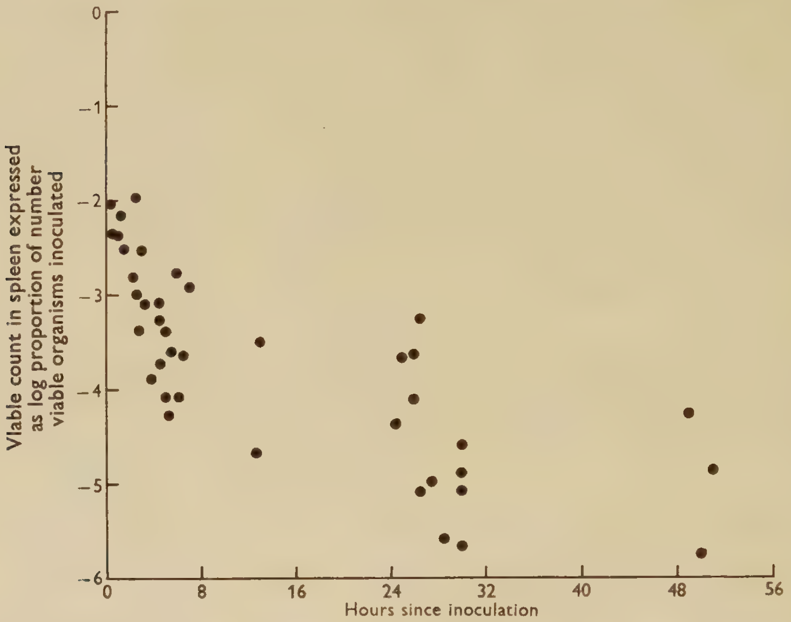

Fig. 6. The number of viable organisms per spleen at various times after inoculation expressed as the log proportion of the number inoculated. The points, each of which gives the count on one spleen, come from unpublished experiments as well as from Tables 2–5. The number of viable organisms inoculated was *c.* 2·5 × 10^8 per mouse.

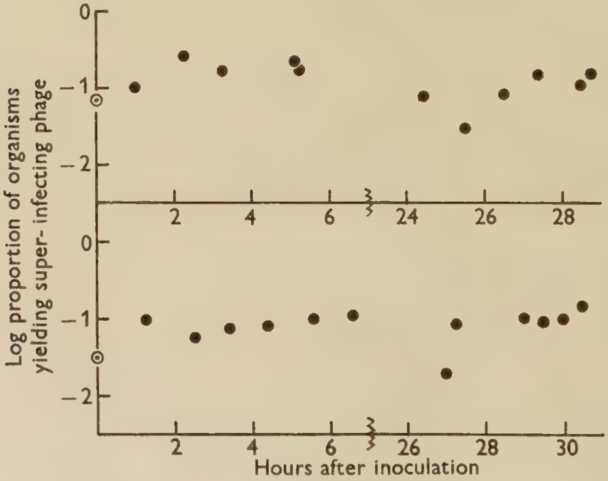

Fig. 7. The proportion of organisms yielding superinfecting phage at various times after inoculation in the experiments shown in Table 2 (upper part of Figure) and Table 3. The points on the ordinate give the average proportion of organisms in the inoculum which yielded superinfecting phage.

Tables 2–5. *The number of viable organisms recovered from the spleen at various times after inoculation and the corresponding proportion of organisms yielding superinfecting phage*

Each Table also gives m, the mean number of superinfecting phage adsorbed per bacterium, and p, the probability per adsorbed particle of being preserved, calculated for the inoculum according to the third hypothesis discussed in the text.

Table 2. $m = 2{\cdot}5$; $p = 0{\cdot}02$

Source of sample	Time between inoculation and sampling	No. viable organisms in spleen	No. plaques* counted on		Proportion of organisms yielding superinfecting phage*
			CR 63	C 600	
Inoculum diluted in BGPW† at 37°	2 min.	—	312	113	0·055
	20 min.	—	340	144	0·047
	40 min.	—	391	144	0·054
Mice given $2{\cdot}76 \times 10^8$ organisms	1·1 hr.	$1{\cdot}1 \times 10^6$	125	115	0·109
	2·3 hr.	$4{\cdot}2 \times 10^5$	101	372	0·272
	3·3 hr.	$2{\cdot}1 \times 10^5$	748	234	0·16
	5·1 hr.	$1{\cdot}3 \times 10^5$	351	160	0·225
	5·3 hr.	$1{\cdot}4 \times 10^4$	262	240	0·218
	24·4 hr.	$1{\cdot}2 \times 10^4$	187	447	0·076
	25·5 hr.	$1{\cdot}4 \times 10^5$	162	1062	0·029
	26·5 hr.	$2{\cdot}3 \times 10^3$	23	58	0·079
	27·4 hr.	3×10^3	42	72	0·145
	28·5 hr.	$6{\cdot}3 \times 10^2$	11	13	0·11
	28·7 hr.	$7{\cdot}1 \times 10^3$	53	52	0·13

* The volume of suspension plated with strain CR 63 often differed from that plated with strain C 600. Consequently, division of the number of plaques does not always yield the proportion shown in the last column.

† BGPW = buffered glucose peptone water

Table 3. $m = 1{\cdot}75$; $p = 0{\cdot}017$

Source of sample	Time between inoculation and sampling	No. viable organisms in spleen	No. plaques counted on		Proportion of organisms yielding superinfecting phage
			CR 63	C 600	
Inoculum diluted in BGPW* at 37°	2 min.	—	664	204	0·0322
	20 min.	—	645	195	0·0332
	40 min.	—	951	352	0·0278
Mice given $2{\cdot}4 \times 10^8$ organisms	1·3 hr.	$7{\cdot}3 \times 10^5$	120	125	0·096
	2·5 hr.	$3{\cdot}4 \times 10^5$	507	45	0·0565
	3·4 hr.	2×10^5	751	336	0·074
	4·4 hr.	$2{\cdot}2 \times 10^5$	703	334	0·07
	5·6 hr.	—	361	429	0·099
	6·6 hr.	$5{\cdot}5 \times 10^4$	738	751	0·115
	27 hr.	—	67	412	0·019
	27·3 hr.	—	10	60	0·081
	29 hr.	—	20	85	0·097
	29·5 hr.	—	9	40	0·093
	30 hr.	—	13	48	0·10
	30·5 hr.	—	21	56	0·145

* BGPW = buffered glucose peptone water.

Table 4. $m = 4{\cdot}8$; $p = 0{\cdot}025$

Source of sample	Time between inoculation and sampling	No. viable organisms in spleen	No. plaques counted on		Proportion of organisms yielding superinfecting phage
			CR63	C600	
Inoculum diluted in BGPW* at 37°	2 min.	—	497	222	0·112
	20 min.	—	520	915	0·113
	40 min.	—	262	146	0·18
Mice given $2{\cdot}3 \times 10^{8}$ organisms	2 hr.	$4{\cdot}5 \times 10^{5}$	672	481	0·14
	2·3 hr.	$2{\cdot}3 \times 10^{5}$	624	210	0·148
	4 hr.	$1{\cdot}9 \times 10^{5}$	668	299	0·112
	4·3 hr.	$6{\cdot}3 \times 10^{4}$	81	174	0·233
	5 hr.	$1{\cdot}3 \times 10^{5}$	365	393	0·169
	5·3 hr.	$3{\cdot}9 \times 10^{5}$	204	456	0·172
	27 hr.	$8{\cdot}3 \times 10^{2}$	2	77	0·106
	27·3 hr.	$2{\cdot}5 \times 10^{3}$	7	35	0·082
	28 hr.	$1{\cdot}4 \times 10^{3}$	3	32	0·038
	28·3 hr.	$1{\cdot}8 \times 10^{3}$	14	39	0·154
	29 hr.	$1{\cdot}5 \times 10^{3}$	1	22	0·187
	29·3 hr.	$1{\cdot}3 \times 10^{3}$	5	43	0·074

* BGPW = buffered glucose peptone water.

Table 5. $m = 3{\cdot}3$; $p = 9{\cdot}1 \times 10^{-4}$

Source of sample	Time between inoculation and sampling	No. viable organisms in spleen	No. plaques counted on		Proportion of organisms yielding superinfecting phage
			CR63	C600	
Inoculum	(see below)*	—	238	728	0·0033
Mice given $2{\cdot}6 \times 10^{8}$ organisms	2 hr.	$3{\cdot}8 \times 10^{5}$	94	645	0·145
	4 hr.	$1{\cdot}9 \times 10^{5}$	141	139	0·102
	25 hr.	$5{\cdot}5 \times 10^{3}$	21	118	0·089

* This was an early experiment in which the inoculum was only cooled to 15° while the mice were injected, instead of to 4° as in later experiments. The proportion of organisms carrying superinfecting phage was measured by u.v.-irradiation of the cooled suspension and was not also determined after incubation of the inoculum in buffered glucose peptone water at 37°. It is supposed that at 15° the bacteria were able to inactivate most of the superinfecting phage while the inoculations were being made (see also curve *B* in Fig. 5, and Sample 3 in Table 1).

into mice because inactivation appears to require conditions suitable for growth (Fig. 2; Table 1) whereas the viable organisms recovered from the spleen do not appear to have divided since inoculation (Tables 2–5; Fig. 7).

The proportion of organisms yielding superinfecting phage might, however, have remained constant for another reason. Cultural conditions are known to affect the rate of lysogenization of a non-lysogenic bacterium by a temperate phage (Bertani, 1958) so that the conditions in the mouse might, conceivably, have increased the rate of lysogenization of *Escherichia coli* strain K12 (λb) by the superinfecting phage from *c.* 0·001 the rate observed *in vitro* (Fig. 4) to *c.* 0·02–0·06, the usual probability/absorbed superinfecting phage of being preserved (shown in Tables 2–5 by the values of p). Phage λhc would then have been present, not as superinfecting phage, but as prophage which would have multiplied synchronously with the bacteria. The proportion of organisms carrying phage λhc would then not have altered, even if the bacteria had divided *in vivo*. This possibility was excluded by plating out colonies obtained from the viable counts in the experiment shown in Table 4. Single colonies from the subcultures were then streaked across the indicator strains, C600 and CR63, and incubated overnight at 37° after u.v.-irradiation for 90 sec. About 40 colonies from the viable count on each spleen were tested, making a total of 504 colonies. None yielded phage λhc although all yielded phage λb. Hence, the superinfecting phage had not entered the prophage state after the bacteria had been inoculated into mice.

The number of organisms induced was usually *c.* 10 % of the viable count and was never greater as might occur if the capacity of the organisms to yield phage after induction was more resistant to inactivation *in vivo* than their ability to form colonies. This may mean that loss of colony-forming ability is due to gross destruction of the bacteria and not to a bactericidal mechanism having a highly specific effect on the organisms.

DISCUSSION

The method described here for determining bacterial division rates appears to be extremely sensitive. Firstly, because N, the initial mean number of superinfecting phage particles preserved/stably superinfected bacterium, can be little more than 1 (Fig. 3), so that each of the early generations causes almost the maximum decrease of one half in the proportion of superinfected organisms. Secondly, because the method can be used successfully when the viable count is relatively very small. Technically the method is simple, the most laborious stage being the washing of the spleen homogenate. This might perhaps be avoided by using a chemical inducer in place of ultraviolet irradiation.

The method rests on two assumptions. The first is that the superinfecting phage will be distributed amongst bacterial progeny in the manner shown in Fig. 1, whatever the interval between divisions. This assumption could be tested by growing the organisms in different media or in a chemostat. The second assumption is that the presence of superinfecting phage does not affect the behaviour of the bacteria *in vivo*. This assumption was justified in the present experiments while the organisms were in the spleen, since it is very improbable that a selective effect would be counterbalanced by just that degree of multiplication needed to keep the proportion of superinfected bacteria virtually constant for 30 hr. In general, however, selection for or against superinfected organisms could only be detected either by observing that the proportion of superinfected organisms steadily rose during the infection or by measuring the division rate by an independent method.

Various other methods were considered before the present one was tested. The only alternative which seemed feasible was the inoculation of bacteria heavily labelled with radioactive phosphorus; the concentration of isotope, and hence the number of generations, being measured by the death rate of the organisms because of isotope decay after recovery from the animal (Fuerst & Stent, 1956). This method is only practicable when, as in the present system, few or no divisions occur. It will be seen that no method will be generally satisfactory that is based on examination of the total (i.e. living + dead) bacterial population because, if killing occurred and produced a large number of dead organisms within a short time of inoculation, subsequent division of the surviving bacteria would produce only a negligibly small change in the composition of the total bacterial population.

The average lethal dose of *Escherichia coli* strain K12 is more than 10^7 organisms by intraperitoneal injection, even when given with mucin (Rowley, 1954) so that this strain is, at the least, highly attenuated for the mouse. Tables 2–5 show that attenuation is here due to the inability of the bacteria to produce viable progeny in their host. This might occur in two ways. First, because the bacteria could not divide, either because of the presence of an inhibitor or because of the absence of an essential bacterial metabolite from the host (Hawking, 1953). (The latter explanation is unlikely since *E. coli*, strain K12 is prototrophic.) Or secondly, because the organisms were able to divide *in vivo* but, in doing so, became phenotypically susceptible to bactericidal factors in the mouse and were invariably killed. An example of such a mechanism is provided by the bactericidal action of serum which will only kill *E. coli* once multiplication has begun (Rowley & Wardlaw, 1958). On this view, the viable counts on the spleen (Fig. 6) give the number of organisms which had not divided since inoculation.

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The Infectivity and Inactivation of Nucleic Acid Preparations from Tobacco Mosaic Virus

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SUMMARY: The infectivity of nucleic acid preparations made by disrupting tobacco mosaic virus with phenol was increased, relative to that of intact virus, by keeping test plants in darkness or at 37° for some time before they were inoculated. The differences in susceptibility to infection of leaves in different physiological states was too great to be explained by differences in the ability of leaf extracts to inactivate nucleic acid preparations *in vitro*. The spontaneous inactivation of the preparations *in vitro* was not prevented by inhibitors of ribonucleases and most additions to the preparations increased the rate of inactivation. Not all the inactivations are readily explicable on the assumption that the minimal infective unit is a pure nucleic acid built up solely from nucleotides. Leaf sap and saliva are reasonably assumed to inactivate because they contain ribonuclease; also, inactivations by formaldehyde phenylglyoxal, and thiaminase in the presence of thiamine, may well reflect reactions with known components of nucleic acids. However, it is less easy to invoke such actions to explain the inactivation by spermine, 'interferon', some oxidizing agents, leaf mitochondria in the presence of some other substances, and the greater rate of inactivation *in vacuo* than in air. Although nucleic acid seems essential for infectivity, it seems prudent to suspend judgement about the precise chemical identity of the minimal infective unit.

Some treatments have long been known to inactivate tobacco mosaic virus (TMV) without appreciably affecting its physical or serological properties (Bawden & Pirie, 1937). While the nucleoprotein particles were thought to be the minimal infective units, this phenomenon remained unexplained, but the discovery that virus fragments consisting predominantly of nucleic acid can be infective (Gierer & Schramm, 1956; Fraenkel-Conrat, 1956) provided an obvious explanation: these treatments presumably have little effect on the surface protein, which is what mainly determines physical and serological properties, but affect more deeply buried structures.

Virus fragments soon lose infectivity in conditions in which the nucleoprotein is stable; a structure essential for reproduction is, therefore, protected in intact particles from many potentially inactivating treatments. A fuller knowledge of the conditions in which TMV fragments become inactivated might throw light on the roles of the different components of virus particles and might help to identify more closely the minimal infective unit. That nucleic acid is an essential part of this unit seems established, but that it alone is infective remains unproven. Preparations of nucleic acid are so much less infective than intact virus containing the same amount of P that analytical studies on the preparations have little bearing on the problem. There is no advantage in postulating additional components gratuitously, but if infectivity is small because preparations are contaminated with much non-infective nucleic acid, the infectivity of those particles that are infective could well depend on components other than those indicated by the conventional nucleic acid formula. If all the original virus particles were infective, inert nucleic acid could arise only because of inactivation during the course of its separation; if this happens, different methods of disrupting the virus might be expected to yield products with different activities. But preparations made by heating, or by exposure to heat or detergents, all have similar infectivities.

An alternative explanation of the disparity between the infectivity of TMV and of nucleic acid preparations is provided by the sensitivity of the nucleic acid towards agents that it meets during the processes of infection. This explanation suggests that infectivity might be stabilized by some treatment or addition, and the reported increase in infectivity when the nucleic acid and protein moieties are recombined (Fraenkel-Conrat & Singer, 1957) may be an example of such a treatment. However, other explanations are possible, for virus protein could also obviously be adding some component necessary for infectivity. To find something other than virus protein that will increase infectivity, whether by stabilizing the fragments *in vitro* or by protecting them from hazards on or in leaves, it is necessary to know more about inactivating conditions so that these can be avoided. We (Bawden & Pirie, 1957*b*) suggested that ribonuclease in the nucleic acid preparations might be the cause of their instability, but inhibitors of this enzyme have not stabilized the preparations and we have had inactivation in many circumstances when it would be unreasonable to invoke ribonuclease as the cause. Indeed, we have found no *in vitro* treatment that increases infectivity and our tests have served only to increase the variety of treatments that destroy it. Current knowledge on the structure of nucleic acid provides no immediately obvious explanation for some of these.

The relative infectivities of nucleic acid preparations and of intact TMV can be changed by changing the conditions under which plants are kept before they are inoculated, for infection by nucleic acid preparations is more affected by the physiological condition of the test plants than is infection by intact virus. This suggests that nucleic acid rarely manifests full infectivity, presumably because much potentially infective material is inactivated in the inoculated leaves. Whether plants are susceptible or resistant, their leaves contain a variety of systems able to inactivate virus fragments and so make unlikely their survival during the necessary interval between inoculation and virus multiplication. Some extracts from plants resistant to infection by nucleic acid inactivated more than did comparable extracts from susceptible plants, but this was not always so. Most of the fragments, therefore, may be potentially infective but be prevented from showing it. If this is the correct interpretation of the relatively low infectivity of nucleic acid preparations, analytical evidence that a 'pure' nucleic acid can infect becomes more cogent in arguments about minimal infective units, but until the interpretation is better established such evidence needs to be used cautiously. For brevity the term nucleic acid will be used in this paper to mean a preparation consisting mainly but not necessarily exclusively of nucleic acid.

METHODS

The TMV preparations used were all made from systemically infected tobacco leaves by a method that does not wholly eliminate ribonuclease but decreases it to a barely detectable level (Pirie, 1956). The virus was precipitated by bringing clarified sap to pH 3·3, redissolving it and precipitating with ammonium sulphate ($\frac{1}{3}$ saturation). After repeating the precipitations with acid and salt, the preparation was incubated for a day at 37° in 0·1 M-citrate (pH 6·5), and again precipitated with ammonium sulphate. It was then dissolved to give a solution containing 10–15 g./l. from which the virus was sedimented three times at 50,000 *g*.

In most of the experiments reported here, nucleic acid made by the modification of the phenol method (Gierer & Schramm, 1956) that we have already described (Bawden & Pirie, 1957*b*) was used. Results with nucleic acid made by fission with warm detergent (Fraenkel-Conrat, 1956) are specifically noted. The great advantage of the phenol method over either the detergent or heating method (Knight, 1956) is that it leaves no intact TMV to be removed by ultracentrifugation. We have not compared products from the three methods in great detail, but they differ no more in infectivity than do separate preparations made with phenol. Usually a large batch was made at one time, divided into many small samples in individual tubes, and stored at −15°, when infectivity is retained for many months. After being kept frozen for some weeks, preparations were often less infective immediately after thawing than they were after keeping for a few hours at 20° or 1 hr. at 37°. This effect, however, was not always obtained, and the stability of different preparations when kept at 20° or 37° also varied somewhat. With all there was much infectivity remaining after 2–3 hr. at 37°, or 20 hr. at 20°, and at 0° there was no obvious inactivation during 20 hr. The preparations were thawed and kept for some hours before they were used in experiments, and samples were usually kept at 0° to act as controls in experiments involving exposure to 37°. Each preparation of nucleic acid was tested to ensure that it was free from residual TMV, by showing that infectivity was rapidly and completely destroyed by incubation with leaf ribonuclease.

Infectivity assays were made in *Nicotiana glutinosa* L. To increase their susceptibility in the summer, plants were raised in shaded glasshouses, and they were usually put in darkness or at 37° for the 24 hr. immediately before they were inoculated. Eight treatments were usually compared in each test, and each was inoculated to at least 9 half leaves. The treatments were distributed systematically over the test plants to compensate as far as possible for differences in susceptibility between different plants and between leaves occupying different positions on the stems. The need for this is even greater when using nucleic acid preparations as inocula than when using intact TMV, for the number of lesions produced by such preparations vary much more from leaf to leaf even when the leaves look similar. We (Bawden & Pirie, 1957*b*) reported that young plants are relatively more susceptible to infection by virus fragments than by TMV and that the upper leaves of plants are relatively more susceptible than the lower ones. This second statement is true when plants are grown in normal glasshouse conditions during summer, but by no means always holds with plants growing in the winter or under shade in summer. Then all leaves may be of comparable susceptibility, or the older ones may be the more susceptible to infection by nucleic acid as they nearly always are when the inoculum is TMV.

Samples under test were usually incubated as 0·1 ml. lots containing 2·5–5 μg. P. They were diluted to 1 ml. with water before inoculating to *Nicotiana glutinosa,* which was done by rubbing the upper surfaces of the leaves with the forefinger wet with inoculum. During the time taken (1–2 min.) to inoculate one sample to 9–12 half leaves, there was no measurable inactivation of the sample, for on average of many counts the last rubbed leaves produced as many lesions as the first. However, after dipping the forefinger in such samples, inoculation must not be too long delayed, for within an hour of a protracted dip of 1 min. the infectivity of samples may be halved, presumably by something secreted through the skin.

RESULTS

Condition of plants and relative susceptibility to TMV and nucleic acid preparations

The number of lesions produced by a given inoculum of TMV varies with the physiological state of the plants. Young, rapidly growing plants produce more lesions than old, slowly growing ones, and plants grown in shade produce more than those grown in bright light; also, with otherwise similar plants, susceptibility to infection is increased by keeping them in darkness or at high temperature for a day or two before they are inoculated (Bawden & Roberts, 1947, 1948; Kassanis, 1952). Differences in the physiological state of test plants have an even greater effect on the numbers of lesions produced by nucleic acid preparations. Table 1 shows that, whereas keeping in darkness or at 37° for two days doubled the numbers of lesions produced by TMV, it increased the number produced by nucleic acid preparations by a factor of 10 or more.

Dilution affects the infectivity of the two kinds of inocula differently. The number of lesions produced by TMV decreases with dilution by less than the factor of dilution, whereas with nucleic acid preparations the numbers fall more steeply. The relative infectivity of the two types of inocula/unit of P, therefore, is not a fixed quantity but depends greatly on the state of the test plants and the concentrations at which the two are compared, and the nucleic acid seems increasingly infective as the susceptibility of plants and the concentration of the inoculum are increased. Plants of different ages, or raised under different light regimes, show even greater differences in their susceptibility to the two inocula than those shown in Table 1. With differently treated plants that differ in their susceptibility to infection by TMV by factors of up to 10, the more susceptible may give hundreds of lesions with a nucleic acid inoculum that produces few or none in the more resistant plants. Even with the most favourable conditions, however, the nucleic acid preparations have never been more than about 1/10 as infective/unit of P as the parent virus.

The factors that control the number of lesions an inoculum produces in a leaf can be grouped into four categories: (1) the number and type of entry points made by rubbing the leaf during inoculation; (2) the ability of the inoculum to withstand inactivation by components of the leaf with which it comes in contact before reaching the site at which it can multiply; (3) the number of potential infection sites; (4) the number of potentially infective particles in the inoculum needed to initiate infection at a site. The number of entry points is unlikely to be affected by the nature of the inoculum, though it well may be by the pre-treatment of the host. Existing knowledge about the relative stability of TMV and its fragments *in vitro* make it probable that the two types of inoculum will differ greatly in sensitivity in the interval between entering the leaf and initiating infection, but there is less basis of confidence about effects of the other two factors.

Table 1. *Effect of preconditioning test plants on the relative infectivity of TMV and TMV nucleic acid*

The TMV and TMV nucleic acid at the specified concentration were inoculated to opposite halves of the same leaves. The control plants were left in normal glasshouse conditions and the treated ones were at 37° or in darkness for the 2 days before they were inoculated; afterwards they were all put in the glasshouse. The results are expressed as the % of the number of lesions produced by the half-leaves of the control plants inoculated with TMV at 5 mg./l.; the actual numbers produced by these 12 half-leaves were 512 and 620.

	Treatment of plants			
Inoculum	Control	2 days at 37°	Control	2 days in darkness
TMV 5 mg./l.	100	160	100	160
1	43	79	28	45
0·2	15	42	3·5	9
RNA 5 mg.P/l.	20	152	4	64
1	3	35	1·5	15
0·2	0·4	10	0·3	3

Kleczkowski (1950) challenged the generally accepted idea that infection results from chance encounters between single virus particles and infection sites of uniform susceptibility, and our results with the two types of inocula seem compatible only with the idea that individual infection sites differ qualitatively. For instance, some allow infection readily by either TMV or nucleic acid, others only with TMV, and changes in the condition of leaves affect behaviour to the two inocula differently. Also, the greater effect of dilution on nucleic acid preparations is apparently not a consequence of inactivation by simple dilution, because samples kept diluted for some hours are no less infective than samples left concentrated and then diluted immediately before inoculation. Hence the chances of a potentially infective dose of nucleic acid infecting at a given site are increased by increasing the total amount of nucleic acid applied, and extra nucleic acid to some extent simulates the effect of combination with virus protein. This again could have two explanations; increasing the amount of nucleic acid may simply increase the chances that one infective unit will survive and infect, or to start the processes that lead to virus multiplication, different numbers of infective units may be needed at different sites. Increasing the total concentration of nucleic acid in inocula by adding yeast nucleic acid to TMV nucleic acid did not increase the number of lesions produced.

Inactivation by systems of the type encountered during inoculation

We have not tried to define the nature of the change in leaves kept at 37° or in darkness that increases their susceptibility to infection, but we have compared various types of extracts from leaves of old and young plants for their ability to inactivate nucleic acid preparations *in vitro*. These comparisons have not explained the differences in susceptibility, but they have shown that there are so many inactivating components in leaves that it becomes increasingly surprising that nucleic acid should ever be infective.

Even before it enters a cell, nucleic acid is at some risk. Water that has lain for 10 min. on an undamaged *Nicotiana glutinosa* leaf as a pool 2–3 mm. deep will inactivate nucleic acid in 4 hr. at 37°. This inactivating power is stable for a month at 0° but is lost on boiling. It has probably little influence on the course of infection because it is not shown unequivocally at room temperature and the potency of washes from leaves of different type is not obviously correlated with their susceptibility to infection. It may be a manifestation of ribonuclease activity but this cannot be demonstrated with certainty, because the washes act strongly only when undiluted and the ability to inactivate nucleic acid is much the most sensitive of the tests for ribonuclease.

Ribonuclease occurs in all the leaf saps that have been studied; it is therefore an obvious potential inactivating agent and it seemed possible that variation in the amount of enzyme in *Nicotiana glutinosa* leaves might account for differences in their susceptibility. Many comparisons, with yeast nucleic acid as substrate, have shown that sap from older, resistant leaves has two to three times the enzyme activity of sap from younger, susceptible leaves. This is a trend in the opposite direction from that found in pea leaves (*Pisum sativum*, Holden & Pirie, 1955) but it is in the direction corresponding to the differences in susceptibility. Quantitatively however the variation in enzyme activity is inadequate to explain the differences in susceptibility unless some *ad hoc* postulates are made about the distribution of ribonuclease in cells of different age.

From its ribonuclease content alone *Nicotiana glutinosa* sap could be expected to inactivate nucleic acid. Table 2 shows that it does, that the extent of inactivation depends on the dilution of the sap and the temperature of the mixture, and that heating sap diminishes its activity. Other similar experiments showed that activity is lost on more prolonged heating at 100° but not on incubation, in the presence or absence of air, at 37° for several days. These results are compatible with ribonuclease action. Diluted sap and ribonuclease (leaf or pancreatic) solutions, with similar abilities to hydrolyse yeast nucleic acid, differed in their ability to inactivate nucleic acid to an extent that seemed to exceed the experimental error. The greater inactivating ability of sap prompted a search for a method of distinguishing between inactivation brought about by ribonuclease and other systems. Although this did not succeed in its main object, it showed that differences in susceptibility are not consistently related to the properties that we have measured *in vitro*.

Table 2. *The inactivation of nucleic acid by sap*

Sap from *Nicotiana glutinosa* was made as described in the text and one sample was heated for 5 min. at 80° and another at 100°. Suitable volumes were then mixed with water to give 0·1 ml. at the specified dilution and mixed with 0·01 ml. of nucleic acid solution containing 50 mg. P/l. After 15 min. at the stated temperatures, the mixtures were diluted to 1 ml. and inoculated to *N. glutinosa*.

Nucleic acid mixed with	Temperature of inoculation (°)	Average number of lesions/leaf
Water	18	41
Sap 1/12·5	0	0
Sap 1/50	0	0·5
Sap 1/200	0	17
Sap 1/50 heated at 80°	0	1
Sap 1/50 heated at 100°	0	14
Sap 1/50	18	0
Sap 1/200	18	2

Table 3. *Inactivation of nucleic acid by sap from leaves of susceptible and resistant leaves*

Sap from susceptible and resistant leaves of *Nicotiana glutinosa* plants was diluted to the extent specified when mixed with the nucleic acid solution. After incubation for the time and at the temperature stated, the mixtures were diluted 1/10 and inoculated to *N. glutinosa*. Leaves on the resistant plants gave no lesions with a nucleic acid preparation that produced more than 200 lesions/leaf on the susceptible plants.

Origin and dilution of sap		Incubation		Average number of lesions/leaf
		Temperature (°)	Time (min.)	
Resistant leaf	1/50	0	10	38
Susceptible leaf	1/3	0	10	32
	1/12	0	10	95
	1/50	0	10	128
Resistant leaf	1/500	18	50	4
Susceptible leaf	1/500	18	50	29
	1/3	Mixed immediately before test		153
Control in water		18	—	240

During the summer, results of the types set out in Table 3 are typical. Sap from highly resistant leaves inactivates nucleic acid five to ten times as rapidly as does sap from susceptible leaves. This factor is probably outside the combined possible uncertainties of the enzyme assays, which showed the sap from resistant plants to contain twice as much activity as that from susceptible plants, and the infectivity tests. There is therefore good reason to think that at least sap from resistant leaves contains an inactivating mechanism in addition to ribonuclease. But even a tenfold difference in inactivating power does not go very far towards explaining the observed differences in susceptibility. Furthermore, although young winter-grown *Nicotiana glutinosa* plants also give many more lesions than old plants when inoculated with the same nucleic acid solution, the ability of sap from old and young leaves in winter to inactivate nucleic acid differs little. Indeed, sap from susceptible leaves was sometimes more potent.

Although the ability of sap to inactivate nucleic acid is largely destroyed by boiling, which suggests that it depends on enzyme-like components, tests with sap dialysates were made because nucleic acid is inactivated by some substances of low molecular weight. All dialysates tested were effective, but as they had only 1/10 to 1/100 the activity of the sap it is unlikely that this type of inactivation can explain differences in susceptibility.

The sap used in the experiments we have described so far was made by grinding one or two half-leaves in a mortar with twice their weight of water (the other half was left on the plant and tested for susceptibility) and centrifuging for 20 min. at 10000 rev./min. after lying for 2 hr. at room temperature. This ageing ensures that chloroplast fragments, mitochondria and similar structures aggregate so that they can be centrifuged out. No experiments were made with these structures from *Nicotiana glutinosa* leaves because they are not a suitable source of enzymically active preparations of mitochondria. Active mitochondrial preparations can be made by grinding young tobacco (*N. tabacum*) leaves at 0° in buffered 0·4 M-sucrose solution and fractionating the extract by differential centrifugation (Pierpoint, 1959). These are still contaminated with chloroplast fragments but examination under the microscope suggests that mitochondria make up about half the material present. They are therefore very different from the crude preparations, made from old infected tobacco leaves, which Bawden & Pirie (1957*a*) found inactivated the Rothamsted culture of tobacco necrosis and some other viruses.

When used sufficiently concentrated, or at 37°, preparations of mitochondria inactivate nucleic acid, probably because they carry a trace of ribonuclease activity. It is of more interest that they also carry enzymes involved in the tricarboxylic acid cycle (Pierpoint, 1959); by analogy with mammalian mitochondria they probably also carry enzymes involved in other syntheses. Some of these are probably integral parts of the mitochondrion but others may well have been absorbed during the processes of fractionation (cf. Pirie, 1959). By choosing conditions in which mitochondria alone have relatively little effect on nucleic acid, the effect of various substrates or stimulants can be studied. Table 4 shows that maleate and ethylenediaminetetraacetate (EDTA) greatly enhance inactivation by the mitochondria, although alone they have little inactivating effect. The effect of citrate is equivocal but some other substrates, notably α-ketoglutarate and isocitrate also promote inactivation by many preparations of mitochondria.

These substances may act in various ways. The tricarboxylic acid cycle substrates, for example, may be converted into substances that act directly on the nucleic acid, whereas EDTA, which is unlikely to act as a substrate, may disorganize mitochondria and release an inactivating agent. To test this last possibility we examined the effect on nucleic acid of supernatant fluids made by centrifuging at 10000 *g* samples of a mitochondrial preparation (10 mg. N/l.) that had been treated in different ways. After 12 hr. in water at 0° the supernatant fluid diminished the infectivity of nucleic acid to about half in 2 hr. at 20°, but the diminution was to less than a tenth when 1 g. EDTA/l. replaced the water. At this concentration EDTA alone does not inactivate. Mitochondria, as has been said, carry a trace of ribonuclease activity in spite of careful fractionation, and this is perhaps stimulated, or more may be liberated, by EDTA. To get significant hydrolysis of yeast nucleic acid it is necessary to use suspensions of mitochondria 200 times more concentrated than those used to inactivate nucleic acid and this hydrolysis is not stimulated by EDTA. It is therefore unlikely that ribonuclease is the inactivating agent but a more detailed study of this phenomenon is not worth while until more is known about leaf mitochondria.

Table 4. *The effect of various agents on the inactivation of nucleic acid by leaf mitochondria*

Mitochondria, made from *Nicotiana tabacum* leaves as described in the text, were suspended at pH 6 at the concentration, and in the fluid, specified, mixed with nucleic acid and the mixture diluted and inoculated to *N. glutinosa* after 2 hr. at 18°.

Concentration of mitochondria (mg. N/l.)	Suspending fluid	Average number of lesions/leaf
0	water	50
30	water	4
7·5	water	25
7·5	2 g. EDTA/l.	2
7·5	0·02 M-citrate	15
7·5	0·02 M-malate	0·5
0	2 g. EDTA/l.	26
0	0·02 M-maleate	20

Inactivation by some enzyme, or enzyme-like, systems

Some of these results, especially those with mitochondria, suggest that the infectivity of nucleic acid depends on some component or structure other than those in the conventional nucleic acid formula. The range of agents that could profitably be tried as inactivators is immense, and it is probable that the use of relatively specific enzyme preparations will provide evidence about the actual nature of the infective agent. This has happened in the past, for the first evidence that protein was a part of potato virus X particles came from a study of the action of proteases (Bawden & Pirie, 1936). Accessibility and general interest have controlled our choice of enzyme preparations with which to start.

Thiaminase. This enzyme is found in Pteridophytes, moulds and bacteria, but so far in only one higher plant. Its ability to catalyse transfer reactions between various heterocyclic compounds related to thiamine (vitamin B_1) is well known but, in the course of reviewing the literature on the enzyme, Kenten (1957*a*) mentioned that it catalyses an action between thiamine and some purines and pyrimidines. This suggests that its normal action *in vivo* may be more general and involve substitutions on nucleic acids.

Table 5 shows that a preparation of thiaminase made from bracken leaves by Kenten (1957*b*) had little inactivating action on its own, but inactivated rapidly in the presence of thiamine. For this action thiamine cannot be replaced by either yeast or thymus nucleic acids, pyridine, piperidine or a number of other bases; nor does thiamine increase the rate of inactivation of the nucleic acid by unfractionated leaf sap, mitochondria or salivary ribonuclease. It would be premature to claim that this effect is caused by thiaminase, but it is clearly not caused by a nuclease in the normal sense of that term.

Table 5. *The effect of thiaminase and thiamine on the infectivity of nucleic acid*

Each tube contained the stated volume of thiaminase solution, in a final volume of 0·1 ml., along with thiamine and nucleic acid. After 4 hr. at 18° they were diluted to 1 ml. and inoculated to *Nicotiana glutinosa*.

Volume of thiaminase solution (ml.)	Concentration of thiamine (g./l.)	Average number of lesions/leaf
0·01	8	2
0·01	2	1
0·01	0·5	18
0·01	0·125	39
0·01	0	76
0·003	2	3·5
0	8	40
0	0	85

Saliva. The presence of many enzymes in this secretion is generally recognized but little attention seems to have been paid to its ribonuclease. The enzyme is important here for two reasons: first to show what hazards a sensitive nucleic acid faces from pipetting and the labelling of tubes, and secondly because a comparison of the inactivating power of saliva and purified pea or pancreatic ribonuclease, with equal ability to hydrolyse yeast nucleic acid, could give evidence about the number of different enzymes that can inactivate. Saliva diluted 1/200 caused 90% inactivation in 40 min. and complete inactivation in 3 hr. In that experiment there was the same inactivation with pea ribonuclease at 0·01 units/ml. (Holden & Pirie, 1955) and this was the ratio of their activities measured by the hydrolysis of yeast nucleic acid. There is therefore no reason to look for enzymes other than ribonuclease to explain the action of saliva.

Interferon. Isaacs & Lindenmann (1957) gave the name interferon to an extract made from chick embryo that had been exposed to inactivated influenza virus. It inhibits the multiplication of several animal viruses but it neither inactivates intact TMV nor inhibits infection by it. The ribonuclease activity of concentrated solutions is barely perceptible with yeast nucleic acid as substrate but they inactivate TMV nucleic acid when diluted 1/1000. Table 6 shows this and that some of the activity withstands boiling. It is difficult to attribute this action to a ribonuclease, and it may not be an enzyme action, but other experiments showed that the extent of inactivation increased with time and it proceeded more rapidly at 18° than at 0°. The preparations may contain more than one component able to act on the nucleic acid, and we have no evidence that any of these is the substance that interferes with the multiplication of animal viruses.

Table 6. *Inactivation of nucleic acid by interferon*

Nucleic acid (3 mg. P/l.) was exposed to the stated dilutions of a preparation of interferon for 1hr. at 18°, when the solutions were diluted and inoculated to *Nicotiana glutinosa.*

Dilution of interferon	Average number of lesions/leaf
1/10	0
1/100	2
1/1000	43
1/10,000	54
Boiled interferon at 1/100	16
Water	158

Effects of oxidizing and reducing agents

The surface of leaf sap becomes brown in a few minutes when it is exposed to air at room temperature. This can be prevented by cooling, excluding air, or adding inhibitors or reducing agents. The normal conditions of inoculation expose exudate from damaged leaf cells generously to oxidation; obviously, this may damage an infecting agent and might account for the low infectivity of nucleic acid. In an attempt to minimize this risk and protect the inoculum during entry, a few leaves were inoculated at 0° or in N_2, but there was no striking effect on the number of lesions produced. A parallel attempt to protect the inoculum by adding neutralized ascorbic acid or cysteine to it showed that these agents diminished rather than increased the apparent infectivity. This, together with the inactivation by leaf dialysates, prompted a study of various oxidizing and reducing systems, though it is unlikely that those studied are actually responsible for the differences in the infectibility of leaves in different physiological states.

Ascorbic acid. At concentrations within the physiological range, ascorbate is not a powerful inactivating agent and it is necessary either to expose nucleic acid to it at 37° or to use prolonged exposures at room temperature. Table 7 shows this, and it is clear that the inactivation can be prevented, or at any rate decreased, by excluding air and by adding diethyldithiocarbamate which inhibits copper-catalysed oxidations. Other experiments showed that the extent of inactivation increases with time and that nucleic acid is not quickly inactivated when added to ascorbate that had undergone aerobic oxidation. These phenomena suggest that the actual inactivating agent is neither ascorbic acid nor its oxidation product, dehydrascorbic acid, but something, presumably an unstable oxidizing agent, produced during the oxidation.

Glutathione and iron. When tested on nucleic acid made by disrupting TMV with sodium dodecyl sulphate, glutathione behaved like ascorbic acid. In

3 hr. at 18° there was complete inactivation by 0·01 M, and 80% inactivation by 0·002 M solutions, in air, whereas in N_2 the extent of inactivation was diminished to 70% and 20%. Nucleic acid made by phenol fission is not systematically inactivated by glutathione under similar conditions. This difference may well arise because of differences in the other components of the system and it is so unphysiological that it has not seemed worth while to explore fully. But attempts to get, by adding $FeSO_4$, conditions in which glutathione would inactivate systematically showed that Fe itself had a surprising ability to inactivate nucleic acid. Thus there is complete inactivation at 20° in 2 hr. with 0·1 mM Fe, and extensive inactivation with 0·02 mM. Exclusion of air diminishes the inactivation and it is diminished by EDTA or 0·02 M-citrate. The amount of Fe in a leaf extract will depend on the extent to which the chloroplasts have been damaged, for they contain most of the leaf Fe. Furthermore, most of the Fe is in organic combination. Published figures for the total Fe content of leaves and leaf fractions (e.g. Hill & Lehmann, 1941) suffice to show only that 0·1 mM would be a high but not impossible value. A few measurements of the colour given by *Nicotiana glutinosa* sap or its dialysate with *o*-phenanthroline show the presence of not more than 0·02 mM-Fe in the inorganic state. Preparations of the intact virus contain about 10 parts of Fe per million (Loring, Al-Rawi & Fujimoto, 1958), but even if all this appears in the separated nucleic acid, solutions of the concentration we used would contain only 2 μM Fe. Again, therefore, we have an equivocal situation. The nucleic acid may contain Fe, but at a level only just relevant to its instability *in vitro*, and the level in leaf extracts is only just relevant to the instability *in vivo*.

Table 7. *The effect of various environments on inactivation of nucleic acid by ascorbic acid*

In two separate experiments, nucleic acid (5 mg. P/l.) was incubated with the stated concentrations of ascorbate at pH 6, with and without air and diethyldithiocarbamate (DIECA); after the stated time the mixtures were diluted and inoculated to *Nicotiana glutinosa*.

Incubation				
Temperature (°)	Time (hr.)	Concentration of ascorbate	Ambience	Average number of lesions/leaf
18	7	0	Air	52
18	7	0·01 M	Air	2
18	7	0·01 M	N_2	47
18	7	0·01 M	1 mM DIECA & air	71
18	7	0	1 mM DIECA & air	32
0	2·5	0	Air	175
37	2·5	0	Air	104
37	2·5	0·01 M	Air	0
37	2·5	0·002 M	Air	0
37	2·5	0	N_2	95
37	2·5	0·01 M	N_2	4
37	2·5	0·002 M	N_2	63

Hydrogen peroxide. As already mentioned, the fact that ascorbic acid inactivates nucleic acid while it is being oxidized suggests that there is an active intermediate and peroxide is the most obvious possibility. At 20° it inactivates intact TMV in a few hours at 5 mM, but in similar conditions 0·5 mM inactivates nucleic acid completely and 80% of the infectivity is destroyed by 0·1 mM. There is no measurable inactivation when the nucleic acid and H_2O_2 are mixed and tested within a few minutes, for the process needs an hour or more; if therefore some similar oxidizing agent is being produced by the other inactivating systems, there is, from experiments of this type, no means of knowing whether the rate of production, the rate of attack, or both are being measured.

Exclusion of air. Excluding air prevents or stops inactivation by the potentially oxidizing systems so far described, but with weaker inactivating agents, or when conditions are so arranged that substantial inactivation demands many hours' exposure, especially at 30°, inactivation is usually greater in samples from which air is excluded. We do not understand what essential factors control this surprising phenomenon; it has been encountered with many different preparations of nucleic acid. Table 8 shows the phenomenon clearly; both with and without citrate, nucleic acid was relatively stable in air at 30° but inactivated when air was excluded. Similarly, at 37°, inactivation was complete without air and partial in air.

Table 8. *The effect of excluding air on the stability of nucleic acid*

Solutions of nucleic acid (5 mg. P/l.) in the environment stated were left for 12 hr. before they were diluted and their infectivities compared.

Nucleic acid in	Temperature (°)	Atmosphere	Number of lesions on eight half leaves
0·02 M-citrate (pH 6)	0	Air	52
	37	N_2	0
	30	N_2	0·5
	30	Air	49
	30	N_2	0
Water	30	Air	35
	37	N_2	0
	37	Air	13

To exclude air, the tubes holding the samples were put in a vessel which was exhausted and refilled with N_2 several times. The process of exhausting the air probably does not affect infectivity because at least 6 hr. without air are needed for significant inactivation, which is not increased by repeated exhaustion with final admission of air for the period of incubation. Contaminants in the N_2 are probably not involved because the phenomenon appears with specially purified N_2, with H_2, and even when the refilling with inert gases is dispensed with but the exhaustion is prolonged. The possibility that anaerobic bacteria are concerned can probably be excluded because inactivation proceeds in 2 g. azide/l. and in the presence of $CHCl_3$. We report these results with some trepidation because it is clear that some important factor is uncontrolled; this is unsatisfactory, but does not affect the fact that inactivation of this type often happens.

Miscellaneous inactivating agents

The effect of various substances on the infectivity of TMV nucleic acid was studied in the hope of finding an environment in which infectivity was stabilized, or that a reaction would be discovered characteristic of a certain type of structure which would give information about the nature of the infective unit. Neither aim was achieved but a few observations are worth recording.

As already noted, Fe inactivates; Zn, Cu, Ni, and Mn at comparable concentrations do not. Their effect depends on the environment in which they are tested. Thus Zn has less effect in 0·01 M-citrate than in water, pyrophosphate or NaCl, but we have not been able to draw any general conclusions from these differences. Ca, Mg and Sr also inactivate preparations free from citrate, but after 2 hr. at 18° inactivation is extensive only in solutions containing these ions at 0·01–0·1 M; ions such as Na and K do not inactivate significantly at this concentration. This type of inactivation is unlikely to have any physiological significance.

Heparin and polygalacturonic acid sulphonate, which inhibit ribonuclease and a few other enzymes (Vandendriessche, 1956; Kaiser & Winkler, 1955), inhibit the activation of nucleic acid by added ribonuclease, but they do not stabilize it against spontaneous inactivation. The commonly used enzyme inhibitors, azide, 8-hydroxyquinoline, iodoacetic acid and diethyldithiocarbamate, likewise had no consistent stabilizing effect. Neither spontaneous inactivation nor inactivation by ribonuclease is particularly sensitive to pH in the range 5–8.

There are many analogies between the inactivation of TMV nucleic acid and of the Rothamsted strain of tobacco necrosis virus, which is sensitive to dilute solutions of formaldehyde, phenylacetaldehyde and phenylglyoxal (Bawden & Pirie, 1957*b*). We have studied the effect of these agents on TMV nucleic acid and confirm Staehlin's (1958) observation that it is much more sensitive to formaldehyde than is intact TMV. Staehlin, using labelled formaldehyde, found that it combined with synthetic polynucleotides; this type of experiment does not therefore give any evidence about the possible role of structures other than nucleotides in infective nucleic acid.

Several bases were used as controls in experiments on the inactivation of nucleic acid by thiaminase in the presence of thiamine and were found to have little effect. Dr R. Markham told us of his (unpublished) observations on the presence of bases related to spermine in preparations of TMV, so, although there is no other evidence for the presence of such bases in higher plants, they seemed to merit trial as inactivators. After exposure for an hour at 20° to 1 mM spermine ($NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$) the number of lesions given by nucleic acid was 5% of the control, and even when the test was made immediately after mixing the number was down to less than half. At 0·3 mM inactivation is considerable and detectable at 0·1 mM. Inactivation is to some extent prevented by salts; 0·3M-sodium acetate or NaCl diminish it and there is little inactivation in 0·02M-citrate. In neutral salt-free solution spermine precipitates TMV nucleic acid as it does yeast nucleic acid (Razin & Rozansky, 1959); this precipitation may be useful in fractionating nucleic acids. This precipitation is prevented by salts but the concentration needed to prevent precipitation under these conditions is only 0·1M which, with acetate, is too little to prevent inactivation; furthermore the precipitate formed in salt-free solution dissolves when salts are added, but infectivity is not thereby restored. Precipitation alone does not therefore account for the phenomenon. The simpler base, spermidine ($NH_2(CH_2)_3NH(CH_2)_4NH_2$) also precipitates and inactivates nucleic acid, but less powerfully and 5mM is needed to get substantial inactivation in an hour. The still simpler base, putrescine ($NH_2(CH_2)_4NH_2$), has no effect under these conditions.

Spermine and spermidine are widely distributed in animal tissues and in yeast so that in some organisms inactivation of this type may be of physiological significance. However, it is unlikely that it does more than provide another example of what may take place in the leaf.

DISCUSSION

Our results emphasize the fragility of nucleic acid preparations made from TMV and provide ample reason for their small infectivity as compared to the intact virus. These results give no direct evidence about the nature of the minimum infective unit, but they suggest that the idea that this unit is nucleic acid may entail some redefinition of that term. If the simple proposition is accepted that tobacco mosaic virus contains only two components, nucleic acid and protein, then infectivity is clearly a property of the nucleic acid, which is protected or stabilized by the protein. However, if nucleic acid is defined more rigidly, not simply by general physical properties, but chemically as a macromolecule containing only nucleotides that are phosphoric esters of ribose or deoxyribose linked to either a purine or pyrimidine, then it cannot be stated confidently that nucleic acid alone is infective. The difficulty arises because chemical analyses and physical criteria of homogeneity cannot establish, as they can with small molecules, that preparations of nucleic acid do not contain, say 1% of some impurity. Analyses may exclude postulated contaminants if these are sufficiently characteristic types of material, but the general proposition that a nucleic acid is more than 99% pure cannot be established. This being so, it is important to consider reasons for supposing that 1% or less of the material may be as significant as the bulk of the preparation for understanding the process of infection. This is an obvious possibility when preparations are poorly infective. Even with preparations of intact TMV many thousands of particles are needed to get a lesion by current methods of inoculation, and nucleic acid is much less infective than intact virus. Hence the observed infectivity could be accounted for by postulating that only one particle in a million carries all the components necessary to make it infective. Although this may be an extreme interpretation, while it is a possible one, physical and chemical measurements are clearly irrelevant to identifying what may be essential components, for the precision of the measurements may be out by a factor of a thousand or more.

The history of work with TMV provides many examples of the need for caution in drawing conclusions about the nature of the infective unit and the significance of its various parts. A cautious approach to the evidence will also help to ensure that the right problem is being studied. The nucleic acid, which today gets so much attention, was at first overlooked, when TMV was described as a globulin (Stanley, 1935), and then dismissed as a contaminant (Stanley, 1937). Even after our evidence (Bawden & Pirie, 1937) for the presence of nucleic acid was generally accepted, analytical work still centred on the protein and claims were made that 'mutations' involved changes in amino acid constitution (Stanley, 1943) and that reversible changes in the protein destroyed and restored infectivity (Ross & Stanley, 1938). It is understandable enough that most attention should be paid to the quantitatively greater component, particularly when many people thought specificity was the prerogative of proteins, but it has long been obvious that, although nucleic acids do not offer such fantastic opportunities for specificity as proteins, they offer enough for any conceivable needs (Bawden & Pirie, 1953).

Popular fashion has now so changed that much effort is being put into attempts to explain specificity in terms of patterns along chains containing only four nucleotides. Ironically, while this is being done, the number of nucleic acid components is steadily increasing, partly because techniques of separating them have improved, but mainly because biochemists are no longer obsessed with the idea that there are only four and so look for more. Two sugars (Smith & Dunn, 1959) and eight bases are now recognized in normal ribose nucleic acids (Littlefield & Dunn, 1958); others can be introduced by supplying organisms with suitable precursors and further research will no doubt reveal still more. It is too early to draw any conclusions about the part played by these other components, but their existence suggests that the effort being put into explaining heredity and some other forms of specificity on the basis of rhythms built from only four nucleotides may be misdirected. The Morse code and most computers work with only two units, but there is no reason for those who are trying to apply 'information theory' to biological synthesis to assume that Nature is similarly parsimonious. In a particle as large as TMV nucleic acid there are obviously ample opportunities for rearrangements among the nucleotides, and it has been apparent since Berzelius introduced the concept of isomerism, 120 years ago, that the arrangement of components is one important part of chemical specificity. However, arrangements may not be everything; specificity may equally depend on exactly what is arranged, and it is important to look beyond recognized nucleotides to see whether specificity, or 'information', may not be carried by other kinds of substance.

There is no *a priori* reason why differences between infective and non-infective nucleic acid should always depend on a break or other change in a chain consisting solely of nucleotides, rather than on a change in some prosthetic group or component, such as carbohydrate, lipid or peptide, attached to it. We have already argued that such components could exist without being detected by current analytical tests used to assess purity and, that being so, the problem needs to be approached indirectly, by finding whether inactivation can always be explained in terms of what is now thought to be the structure of nucleic acid. When it cannot, two courses are open. Comparable treatments can be applied to other nucleic acids or, preferably, synthetic polynucleotides (Grunberg-Manago & Ochoa, 1955), to see whether they produce any changes that might explain the inactivation by affecting nucleotide structure or arrangement. Secondly, the concept of nucleic acid can be broadened to accommodate the inactivation, and the idea accepted that something with the general physical and chemical properties of a nucleic acid may have attached to it a highly relevant prosthetic group. The concept is already established with proteins; haemoglobin, for example, is called simply a protein, but it is the haemin and not the part of the particle composed of amino acids that is usually held responsible for its behaviour in the presence of O_2 and CO. Similarly, some of the conditions in which TMV nucleic acid inactivated in our experiments suggest that there may be important parts of the infective unit other than nucleotides.

The arrangement or constitution of the predominant protein of the virus clearly plays little or no part in determining specificity, but it is no less an essential component because of this. It is highly effective in protecting the fragile infective parts against many lethal environments. The great stability of tobacco mosaic seems to lie either in the structure of its protein or in the manner in which protein and nucleic acid are combined. Although the proteins of many other viruses do not denature any more readily, these viruses nevertheless inactivate in conditions where TMV is stable; the inactivation, for example, of the Rothamsted tobacco necrosis virus by mitochondria, gentle heat or oxidation, leaves serological activity and general physical behaviour unimpaired, suggesting that such inactivating agents act on the nucleic acid. The separated nucleic acid of TMV also inactivates rapidly with similar treatments, so that in the intact virus particles it seems better protected than is the nucleic acid of viruses with spherical particles.

There is now much circumstantial evidence compatible with the idea that virus particles fragment in the initial stages of infection. If this happens differences in the susceptibility to infection of different plants, or of different leaves of one plant, could depend on the relative abilities of the cells to inactivate the infective units when these are freed from their protecting protein. We started our experiments hoping to find, by *in vitro* tests with nucleic acid preparations, systems that might explain the differences in susceptibility. Although this hope was not realized, our results show that, if nucleic acid does become free *in vivo*, it is at hazard from a variety of potential inactivators. However, as none of the inactivating systems we used seemed adequate to explain the differences in susceptibility we encountered, we studied none in great detail, and we report our results to illustrate the possibilities rather than to demonstrate what actually happens *in vivo*. Our failure to get any direct evidence on the important problem of variation in susceptibility does not exclude this mode of approaching the problem; it may mean only that we were using virus fragments different from those into which the virus separates early in the infection process, or were exposing the fragments to the wrong inactivating systems.

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The Inhibition of *Corynebacterium diphtheriae* and other Gram-positive Organisms by *Staphylococcus aureus*

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SUMMARY: A large collection of *Staphylococcus aureus* strains of human origin was examined for ability to inhibit the growth of *Corynebacterium diphtheriae* on the surface of solid media. Inhibition with the formation of a sharply-defined zone was produced by members of *S. aureus* Type 71, by certain closely-related strains, and by very few others. A small number of strains gave a wider hazy zone of inhibition. Both types of zone were produced by agents which diffused through agar, but not through cellophan, and killed *C. diphtheriae*. The two sorts of agent differed in their spectrum of activity for other corynebacteria. Strains of *S. aureus* which produced sharp zones of inhibition of *C. diphtheriae* also had a weak inhibitory activity against strains of *S. aureus* which were non-inhibitors or producers of hazy zones, but not on others which produced sharp zones. Ability to give rise to hazy zones persisted indefinitely in culture, but ability to produce sharp zones was regularly lost in culture after a few months. Loss of activity was accompanied by a widening of the susceptibility of the staphylococci to lysis by typing phages.

It has been known for many years that some staphylococci are able to inhibit the growth of *Corynebacterium diphtheriae*. Dujardin-Beaumetz (1932) showed that staphylococci which possessed this property were also active against a number of other Gram-positive cocci, and that their inhibitory effect was only demonstrable on solid media. Duliscouët (1935, 1939, 1945) confirmed the inhibitory action of some staphylococci on corynebacteria and in addition described a stimulating effect produced by others. He showed that inhibition was due to the production during growth of a substance which diffused out into the medium. Both of these workers used a technique of 'simultaneous antagonism' (Gratia, 1946) in which spots or streaks of staphylococci were deposited on a solid medium immediately after its surface had been evenly sown with an inoculum of corynebacteria. Several later workers investigated the inhibitory activities of Gram-positive organisms by using more sensitive methods similar to those which Gratia & Fredericq used for the study of colicines (see Fredericq, 1948). In these 'deferred antagonism' techniques (Gratia, 1946), the strain being tested for inhibitory activity was grown on a solid medium for one or more days before the strain to be inhibited was inoculated. In this way a much greater number of active strains were detected, and it became apparent that a complex pattern of antagonisms existed not only between staphylococci and corynebacteria (Jennings & Sharp, 1947) but also between staphylococci and other staphylococci (Fredericq, 1946; Halbert, Swick & Sonn, 1953) and between corynebacteria and corynebacteria (Thibaut 1949; Terrasse & Sohier, 1954). The possession of inhibitory activity appeared, however, to be distributed in a haphazard fashion among members of each of these bacterial groups, and the production of a particular inhibitory substance was in no instance characteristic of a particular species or type of organism.

Parker, Tomlinson & Williams (1955) found that *Staphylococcus aureus* strains belonging to a well-defined type, which could be identified by susceptibility to a single phage (Type 71), were closely associated with *impetigo contagiosa*, and to a lesser extent with superficial vesicular skin infections of new-born infants, but were rarely seen in deep lesions. Many years before, A. J. H. Tomlinson (unpublished investigation) had observed that over half of the strains of *S. aureus* from impetigo lesions, and a much smaller proportion from other sources, inhibited the growth of corynebacteria. He used a simultaneous antagonism technique in which loopfuls of staphylococcal broth cultures were superimposed on a freshly-inoculated lawn of a diphtheroid on a blood agar plate: after overnight incubation, the areas of staphylococcal growth were surrounded by zones of inhibition of the corynebacterium. When *S. aureus* Type 71 strains were tested in this way, it was found that 90% of them inhibited the growth of a strain of *Corynebacterium diphtheriae*; most other strains of *S. aureus* showed no inhibitory action. It was also noticed that staphylococci could give rise to one of two quite different sorts of inhibitory zone on lawns of *C. diphtheriae*. The zones formed by *S. aureus* Type 71 strains were always sharply defined and rather narrow (2–4 mm. radius), but those formed by a number of other strains were wider (more than 5 mm. radius) and hazy at the periphery. Later investigations (Parker, 1958) showed that most of the few strains, other than members of Type 71, which produced narrow sharp zones had phage patterns not very different from that of Type 71, had a number of other '71-like' characters, and were also associated with impetigo and other superficial vesicular skin infections.

The present communication summarizes observations made in the course of this work on the properties of these inhibitory agents, and on the associated changes which take place in a strain of *Staphylococcus aureus* when the ability to inhibit *Corynebacterium diphtheriae* is lost.

METHODS

Organisms

Coagulase-positive staphylococci. Strains (2032) of coagulase-positive staphylococci isolated in the laboratory or received from elsewhere during 1953–57 were tested for ability to inhibit the growth of *Corynebacterium diphtheriae*, and were phage typed by the method described by Anderson & Williams (1956). All strains lysed by phage 71 either at the routine test dilution (RTD) or at ×1000 the strength of the routine test dilution (1000 × RTD), but not lysed by any other phage at the RTD, were included in Type 71.

Coagulase-negative staphylococci. Twenty-two strains of coagulase-negative, catalase-positive, Gram-positive cocci isolated from nasal swabs or from skin lesions were used in various tests for susceptibility to the action of *Staphylococcus aureus* cultures.

Corynebacteria. The strain used in the routine inhibition test was a nitrate-negative, non-toxigenic strain of *Corynebacterium diphtheriae mitis*, No. 51 in the collection kept in this Laboratory. Thirty-nine other corynebacteria, listed in Section B2, p. 466 were used in inhibition tests with a limited number of strains of *Staphylococcus aureus.*

Other organisms. Twelve strains of haemolytic streptococci (4 each of Groups A, C and G), and 2 strains of pneumococci isolated from routine material, were used in various inhibition tests.

Methods used in the study of inhibition on solid media

(*a*) *Simultaneous antagonism* (Gratia, 1925, 1946). In these methods, the active strain was deposited on the surface of an agar medium over which the sensitive (or passive) strain had been recently spread. Such a procedure was only of value when the inhibitory substance was powerful and was produced promptly.

(i) *The routine inhibition test.* Before use, the test strain of *Corynebacterium diphtheriae* had been subcultured several times at daily intervals on blood agar. In earlier experiments the surface of a blood agar plate was sown with *C. diphtheriae* by spreading a loopful of growth from a previous blood agar plate as evenly as possible over the whole surface, but later it was found that a more even sheet of growth of the correct thickness could be obtained by flooding the plate with a broth emulsion of growth from blood agar, roughly standardized to the opacity of Brown's No. 3 tube. As soon as the surface of the plate was dry, small loopfuls of overnight broth cultures of staphylococci were placed in rows on it. After overnight incubation at 37° the corynebacterium had grown as an even confluent sheet, with zones of inhibition around some of the circular areas of staphylococcal growth. The radius and other characters of the zones were recorded. The inhibition test was considered positive (DI+) when the zone was 1 mm. or more in radius. The effect of variations in the technique of the test will be discussed on pp. 462–4.

(ii) *The overlapping drop technique.* This method was based on the cross-titration procedure of Rosebury, Gale & Taylor (1954) in which pairs of overlapping drops of broth cultures were placed on a solid medium. Drops were deposited with a standard loop on a blood agar plate: the second drop of each pair was added as soon as the first had dried and overlapped the first by approximately $\frac{1}{3}$ of its area. Plates were examined for inhibition extending beyond the area of overlap after 18 hr. at 37°. This was a useful method for examining each of a group of cultures for inhibition of, or by, every other member of the group.

(*b*) *Deferred antagonism* (Gratia, 1946). In these methods the active strain was allowed to grow on a solid medium for 1 or more days before the sensitive strain was inoculated. They were designed to detect inhibitory substances weaker than those revealed by techniques of simultaneous antagonism.

(i) *Direct streaking methods.* The active strain was grown as a spot at the centre of a plate. After incubation, passive strains were streaked up to the primary growth as a series of radial lines. It proved difficult to inoculate the passive strains close enough to the primary spot to detect weak inhibition, but the method was useful for measuring the rate of diffusion of inhibitory agents through agar.

(ii) *Methods employing chloroform.* Gratia (1925) found colicines to be resistant to prolonged contact with chloroform, and Fredericq (1948) grew active strains as central spots, killed them with chloroform, and inoculated the whole surface with a passive strain. We used this method in earlier experiments but later adopted a more convenient variation which had been developed in this Laboratory for the colicine typing of *Escherichia coli* 055 and *Shigella sonnei* (Shannon, 1957; Abbott & Shannon, 1958). A streak of growth of the active strain was inoculated across the centre of a blood agar plate and incubated at 37° for 48 hr. The Petri dish was then placed lid downwards on the bench and a circular piece of filter paper inserted and saturated with chloroform. After 1 hr. the growth was scraped to one end of the plate with the edge of a clean glass slide and removed, together with a small piece of agar. More chloroform was then added to the paper in the lid and the plate left over it for a further 1 hr. Then the paper was removed and the plate exposed to the air face downwards for 2 hr. Loopfuls of broth cultures of passive strains were then streaked across the medium at right angles to the position formerly occupied by the active strain. After overnight incubation at 37° the plates were examined for evidence of inhibition of the passive strains near the centre of the lines of growth.

(iii) *Cellophan method.* Autoclaved sheets of commercial cellophan were placed on the surface of blood agar plates and allowed to dry. A spot of staphylococcal broth culture was placed in the centre of each cellophan sheet and the plates were incubated for various periods at different temperatures. The cellophan sheets were then removed, the surface of the medium was flooded with a broth suspension of *Corynebacterium diphtheriae*, and the plates incubated overnight at 37°.

(iv) *Filter-paper method* (Gratia, 1946). When it was desired to detect inhibitory substances not able to pass through cellophan, a sheet of filter paper was placed on the surface of a blood agar plate and a layer of molten nutrient agar poured on top. A drop of staphylococcal broth culture was placed in the centre of the plate, which was then incubated. The top layer of agar was then stripped off with the filter-paper, so removing all the bacteria, and the underlying blood agar was flooded with a suspension of *Corynebacterium diphtheriae.*

RESULTS

Inhibition of Corynebacterium diphtheriae *No.* 51 *by* Staphylococcus aureus

Distribution of inhibitory properties among Staphylococcus aureus *strains from various sources.* The routine inhibition test was used to study the distribution of inhibitory properties among a large collection of *Staphylococcus aureus* strains of human origin. It was not a highly sensitive method, but it gave very clear cut results and the distinction between the sharp and the hazy type

Table 1. *Inhibition of* Corynebacterium diphtheriae *by strains of* Staphylococcus aureus

The figures in each 'box' are:
1 . . number of strains tested
. 2 . number giving a sharp zone of inhibition (DI+S)
. . 3 number giving a hazy zone of inhibition (DI+H)

Phage group	Superficial lesions: Impetigo	Superficial lesions: Newborn infants in hospital	Superficial lesions: Older patients (not impetigo)	Deep lesions	Nasal swabs	Other	Total
I	42	167	38	103	76	19	445
	1	2	2	0	4	0	9
	3	1	1	0	2	2	9
II Type 71	379	66	11	4	17	57	534
	354	64	9	2	16	54	499
	0	0	0	0	0	0	0
Other	68	27	17	60	55	9	236
	17	4	2	0	0	3	26
	0	0	0	0	0	0	0
III	68	89	102	51	39	61	410
	6	1	2	0	1	1	11
	0	0	2	0	0	0	2
Not typable	75	63	37	35	66	21	297
	15	1	0	0	4	1	21
	11	0	3	0	6	5	25
Unclassifiable	20	19	11	15	37	8	110
	1	0	0	1	2	0	4
	3	0	1	0	11	1	16
Total	652	431	216	268	290	175	2032
	394	72	15	3	27	59	570
	17	1	7	0	19	8	52

of zone was easily seen (Pl. 1). Table 1 summarizes the results of tests on 2032 strains, showing the numbers giving sharp zones (DI + S) and the number giving hazy zones (DI + H) in the various phage groups, and from different sources. The main points of interest are displayed in Figs. 1 and 2.

Over 93% of all *Staphylococcus aureus* Type 71 strains were DI +, and all positive reactors formed sharp zones. Most of the small number of other DI + S strains were either members of Group II other than Type 71 or were non-typable, and most had been isolated from impetigo or from vesicular skin lesions of new-born infants. As described elsewhere (Parker, 1958), these strains had other '71-like' cultural characters, and were in the main egg-yolk negative, producers of opacity on serum agar plates and penicillin resistant.

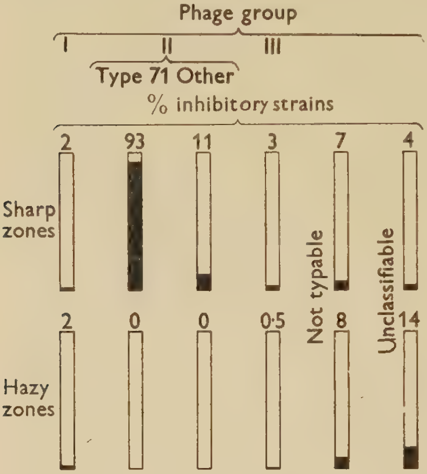

Fig. 1

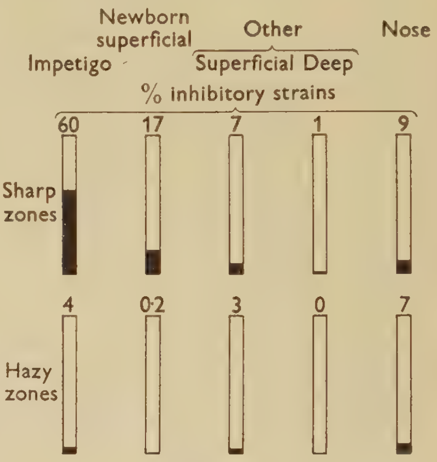

Fig. 2

Fig. 1. Inhibition of *Corynebacterium diphtheriae* by *Staphylococcus aureus*. Percentage of inhibitory strains in phage groups is shown.

Fig. 2. Inhibition of *Corynebacterium diphtheriae* by *Staphylococcus aureus*. Percentage of inhibitory strains from different sources.

Only 2·6% of all strains formed hazy zones. Some DI + H strains occurred in all phage groups except II, but the majority were either unclassifiable or non-typable; those which could be typed showed a wide variety of patterns. They were found most commonly in nasal swabs from normal people and in superficial lesions in which they appeared to be secondary invaders. Some were penicillin sensitive and some resistant and most, but not all, were egg-yolk positive.

A few tests were made with the more sensitive Gratia-Fredericq chloroform method, with incubation of the staphylococcal cultures for 48 hr. before *Corynebacterium diphtheriae* was added. These tests revealed the presence of numerous weaker inhibitory agents produced by strains other than those positive in the routine test. Their distribution did not appear to have any relation to the other characteristics under investigation, and these strains were not investigated further.

Nature of the inhibitory action of Staphylococcus aureus *on* Corynebacterium diptheriae

The inhibitory action of *Staphylccoccus aureus* on *Corynebacterium diphtheriae* was studied in experiments in which they were grown together on solid medium. The fact that the zone of inhibition extended for several mm. beyond the staphylococcal macrocolony suggested that inhibition was due to diffusion of some substance formed by the active staphylococcal strain during growth. Inhibitory activity was also demonstrated with DI+S and DI+H strains, in absence of living staphylococci, either on plates which had been treated with chloroform or in the depths of an agar sheet on the surface of which staphylococci had been grown and later removed by Gratia's filter-paper method. In both types of experiment the inhibition of *C. diphtheriae* extended 0·5–1 cm. beyond the area originally occupied by the staphylococcus. The rate of diffusion of inhibitory agents was studied by spot-inoculating a staphylococcus at the centre of a plate, and streaking a loopful of a broth suspension of *C. diphtheriae* up to it on each of several successive days. There appeared to be no gross difference in the rate of diffusion of the inhibitory agents of DI+S and DI+H staphylococcal strains after the first day; zones of inhibition up to 2·5 cm. in width were seen when the corynebacterium was inoculated after 5 days' incubation of the plate. None of the inhibitory agents tested was able to diffuse through cellophan.

Blood agar was chosen as the medium for the routine test, but inhibition occurred also on a number of other media. Good zones were formed on serum agar, but the distinction between sharp and hazy zones was not easily seen. *Corynebacterium diphtheriae* No. 51 grew poorly on plain agar, but with diphtheroids which were able to grow well in the absence of blood there was little difference in the size of the zones formed on blood agar or on plain agar. Inhibition occurred also on plates incubated anaerobically.

The outer edge of the sharp zone seen in the routine inhibition test was formed by an almost vertical wall of growth of the corynebacterium; the floor of the inhibition zone appeared at first sight to be devoid of bacterial growth but when it was touched lightly with a wire it was seen to be covered with a fine film. The inner 2–3 mm. of a hazy zone was similarly covered with a fine film and beyond this the growth became progressively thicker as it merged into the surrounding lawn. Microscopic examination of the fine film of growth showed the presence of numerous bacilli. Although *Corynebacterium diphtheriae* No. 51 normally had a typical *mitis* morphology on blood agar, these organisms in the fine film were shorter, more pointed, stained very poorly, and were beaded. Subculture from the fine film was invariably sterile. In the sharp inhibition zones no growth was obtained until the outer, perpendicular wall of the zone was touched: in the hazy zones, subcultures from the inner 3 mm. were also sterile, but small numbers of organisms could be grown from the intermediate areas of partial inhibition. On continued incubation of plates beyond the first day there was no further extension of the zones, and no colonies of resistant organisms appeared in them.

In the routine inhibition test plates were flooded with a thick suspension of *Corynebacterium diphtheriae*. This gave rise to an even confluent sheet of growth in which the distinctive characters of the sharp and the hazy type of zone could be well seen. Further increases in the concentration of the suspension had little effect on the final result, but decreases in concentration resulted in a progressive increase in zone size.

With DI + S staphylococcal strains, each 100-fold decrease in concentration of the corynebacterium increased the zone radius by about 1 mm., up to a maximum of 6–7 mm., and the character of the zone was unaltered; but with DI + H staphylococcal strains the effect of decreasing the size of inoculum of *C. diphtheriae* was to alter the character of the zone. The total diameter was little affected, but there was a progressive decrease in the width of the intermediate hazy area. Thus, when the inoculum had been so decreased as to give a semi-confluent lawn of corynebacterium, there was little difference in the appearance of the zones produced by DI + S and DI + H staphylococcal strains.

The size of the initial inoculum of staphylococcus was of little importance. An increase beyond that necessary to give rise to a confluent macrocolony did not result in any increase in the width of zone, and a decrease in inoculum size which resulted in semiconfluent growth, or even discrete colonies, was associated with only a small decrease in zone size. Indeed recognizable zones were formed round well-separated single colonies.

Unsuccessful attempts were made to demonstrate the inhibitory agent in extracts of agar media on which active staphylococci had grown. A number of DI + S and DI + H strains were grown on semi-solid nutrient agar for 18 hr. Fluid was extracted by freezing and thawing and was freed from living staphylococci by heating for 30 min. at 65° or by passage through sintered glass filters, and tested for activity on lawns of *Corynebacterium diphtheriae* in punch-holes or Heatley cups. The agent was unsuccessfully sought in the bottom layer of plates prepared by Gratia's filter-paper method. The agar was extracted with broth, with aseptic precautions, and tested for inhibitory action without further treatment. Broth cultures of active staphylococci grown aerobically at 25° and 37° for periods up to 6 days were tested for activity after heating at 65° for 30 min., or after filtration or treatment with chloroform. Undiluted broth did not prevent the growth of as few as four *C. diphtheriae* organisms. However, small numbers of living staphylococci had a pronounced inhibitory effect on the growth of *C. diphtheriae* in liquid media. In one experiment *C. diphtheriae* No. 51 could not be recovered from 1 loopful of blood broth 24 hr. after it had been inoculated along with an active staphylococcus in a ratio of $10^5:1$.

Inhibition of other organisms by Staphylococcus aureus

Other corynebacteria. Dujardin-Beaumetz (1932) and Duliscouët (1935) both found that active staphylococci inhibited the growth of all the corynebacteria they examined. In the earlier stages of the present work several strains of *Corynebacterium diphtheriae* were tried, and all appeared to be equally susceptible to the action of *Staphylococcus aureus* Type 71. Later Dr H. G. M. Smith

(Bradford) sent us a corynebacterium said to be susceptible to inhibition by Type 71 but not by other staphylococci. This Bradford diphtheroid gave sharp zones of inhibition, 2 mm. or more wide, with all DI + S staphylococcus strains and was not inhibited by most DI + H strains. It had been isolated from an ear swab and was a short broad bacillus forming flat, whitish, opaque and very friable colonies with lobate edges and a dull granular surface. Diphtheroids resembling this strain culturally are commonly grown from swabs of ear and vaginal discharges. We isolated 9 such strains and found that they all behaved similarly to the Bradford diphtheroid in the inhibition test, though they were not a homogeneous group biochemically.

A collection of 40 corynebacteria, which included representative strains of *Corynebacterium diphtheriae*, some atypical diphtheria bacilli, one or more strains each of *C. ulcerans*, *C. ovis*, *C. pyogenes*, *C. haemolyticum*, *C. equi*, *C. hofmanni*, *C. xerose*, *C. fimi* and 19 unnamed diphtheroids, was next tested for inhibition by *Staphylococcus aureus*. The Bradford diphtheroid and 9 strains selected for their cultural resemblance to it were included. Each corynebacterium was tested for inhibition by a number of *S. aureus* strains previously found to be active against *C. diphtheriae* No. 51 by the routine technique. As this took several weeks to carry out, each staphylococcus was tested against *C. diphtheriae* No. 51 at the beginning and at the end of the series, and only those which gave rise to a zone of inhibition at least 1 mm. wide on both occasions and which showed much the same zone size in the two tests will be considered. Successful tests were carried out with 16 *S. aureus* DI + Type 71 strains, with 18 others giving sharp zones and with 30 giving hazy zones on *C. diphtheriae* No. 51. The results are summarized in Table 2.

(i) Five strains of *Corynebacterium diphtheriae mitis* (including No. 51), one *gravis*, and three aberrant *gravis*-like organisms were inhibited by all, or nearly all, of the staphylococcal strains. The characteristic differences between the sharp and hazy type of inhibition zone was apparent in all instances. The growth of none of the corynebacteria in the group was stimulated by any staphylococcus.

(ii) Eleven strains were inhibited by a proportion of staphylococci in each group: nine by all or nearly all. With the exception of *Corynebacterium ovis*, these strains were diphtheroids which grew either as a thin dry sheet in which the zones were wide and usually surrounded by a zone of enhancement of growth, or as a thick creamy growth in which zones were narrower and rather ill-defined. In none could any distinction between sharp and hazy zones be made out.

(iii) Eleven strains (including the Bradford diphtheroid) were inhibited by all or most Type 71 staphylococcal strains, but by few of the hazy zone producers. All nine of the diphtheroids selected for their colonial resemblance to the Bradford diphtheroid were in this group. All except one of them also showed considerable enhancement of growth around the zone of inhibition. The only other strain falling into this group was one of the three representatives of *Corynebacterium xerose*.

(iv) Six strains were more often inhibited by hazy-zone-producing staphylococci than by others. The most characteristic members of this group were

Table 2. *Inhibition of other corynebacteria by strains of* Staphylococcus aureus *active against* Corynebacterium diphtheriae *strain* 51

S = Strains giving sharp zone on strain 51
H = Strains giving hazy zone on strain 51
MPHL = Manchester Public Health Laboratory
NCTC = National Collection of Type Cultures
BPHL = Bradford Public Health Laboratory

			No. of strains of *S. aureus* active		
			S		H
No.	Description	Origin	Type 71	Other	
(i) 1	*C. diphtheriae mitis*	MPHL (No. 51)	16	18	30
2	*C. diphtheriae mitis*	MPHL	16	18	30
3	*C. diphtheriae mitis*	MPHL	16	9	30
4	*C. diphtheriae mitis*	MPHL	16	8	30
5	*C. diphtheriae mitis*	MPHL	16	7	30
6	*C. diphtheriae gravis*	MPHL	15	8	22
7	*C. diphtheriae* aberrant	MPHL	16	18	30
8	*C. diphtheriae* aberrant	MPHL	15	6	12
9	*C. diphtheriae* aberrant	MPHL	16	8	30
(ii) 10	*C. ovis*	NCTC 3450	15	5	9
11	*C. hofmanni*	NCTC 8338	13	8	26
12	*C. xerose*	NCTC 7238	16	13	8
13	*C. hofmanni*	NCTC 231	11	13	30
14	*C. xerose*	NCTC 8481	14	15	19
15	*C. fimi*	NCTC 7547	16	16	30
16	*C.* sp.	NCTC 7491	16	16	24
17	*C.* sp.	NCTC 764	13	12	30
18	*C.* sp.	NCTC 8484	16	17	29
19	*C.* sp.	NCTC 934	11	13	30
20	*C.* sp.	MPHL	14	6	27
(iii) 21	*C.* sp. 'Bradford'	BPHL	16	18	1
22	*C.* sp. 'Bradford-like'	MPHL	15	9	3
23	*C.* sp. 'Bradford-like'	MPHL	14	5	0
24	*C.* sp. 'Bradford-like'	MPHL	15	6	0
25	*C.* sp. 'Bradford-like'	MPHL	16	13	0
26	*C.* sp. 'Bradford-like'	MPHL	15	8	3
27	*C.* sp. 'Bradford-like'	MPHL	15	10	2
28	*C.* sp. 'Bradford-like'	MPHL	15	12	4
29	*C.* sp. 'Bradford-like'	MPHL	16	18	0
30	*C.* sp. 'Bradford-like'	MPHL	16	15	1
31	*C. xerose*	NCTC 7423	16	8	0
(iv) 32	*C. diphtheriae intermedius*	NCTC 3987	7	3	28
33	*C. diphtheriae intermedius*	NCTC 3988	2	2	11
34	*C. ulcerans*	MPHL	0	0	20
35	*C. pyogenes*	NCTC 5224	0	0	24
36	*C. equi*	NCTC 1621	0	3	28
37	*C.* sp.	MPHL	4	1	25
(v) 38	*C. haemolyticum*	NCTC 8452	0	0	0
39	*C.* sp.	NCTC 8483	0	0	1
40	*C.* sp.	MPHL	2	2	0

Corynebacterium ulcerans, *C. pyogenes*, *C. equi* and one diphtheroid producing a creamy growth on blood agar. The zones were all rather narrow or ill-defined. The two strains of *C. diphtheriae intermedius* produced wide zones that were so faint as to be almost invisible.

(v) Three strains, including *Corynebacterium haemolyticum*, were completely or almost completely insusceptible to inhibition.

Staphylococcal strains other than members of Type 71 which produced sharp zones of inhibition of *Corynebacterium diphtheriae* No. 51 were, on the whole, less active against other corynebacteria than Type 71 strains. It was not possible by inspection of the results to define any sub-patterns of activity that might be useful in classification.

Other Gram-positive organisms. As a first step, 16 strains of *Staphylococcus aureus* including both DI+S and DI+H strains, and representatives of all phage groups, were tested for mutual activity by a simultaneous antagonism technique, the overlapping drop method of Rosebury *et al.* (1954). Also, representative DI+S and DI+H strains were tested against 46 unselected *S. aureus* strains, and 15 coagulase-negative staphylococci. No evidence of inhibition was obtained, suggesting that powerful agents active on Gram-positive cocci were not very common in *S. aureus.* However, when the more sensitive deferred antagonism technique of Abbott & Shannon (1958) was used, evidence of inhibition of some staphylococci by others was obtained. All the active strains were DI+S and they inhibited all the DI− and DI+H strains against which they were tested. The results of a typical experiment are shown in Table 3. Some of the reactions were quite weak and in most cases some resistant colonies were visible in the areas of inhibition. Further experiments showed that several DI+ '71-like' members of phage Group II had a similar effect on other staphylococci. It seemed possible, therefore, that the same agent might be responsible for both the sharp-zone inhibition of *Corynebacterium diphtheriae* and for the inhibition of other *S. aureus* strains. Some support for this was obtained from an experiment in which a DI+S Type 71 strain and a DI− variant derived from it were tested for mutal inhibition: the DI− variant was inhibited by the DI+ parent strain, but itself had no inhibitory activity.

Experiments were also carried out, using Abbott & Shannon's technique, in which 14 strains of *Staphylococcus aureus* (including 4 DI+S and 5 DI+H strains) were tested for activity on 7 coagulase-negative staphylococci, two pneumococci and 12 haemolytic streptococci (4 each of groups A, C and G). Each *S. aureus* strain gave evidence of inhibition of one or more organism, but the pattern of activity was extremely complex, and the DI+ strains did not appear to behave in a uniform fashion.

Loss of power to inhibit corynebacteria

Early in the investigation it was observed that *Staphylococcus aureus* Type 71 strains lost their ability to inhibit corynebacteria on storage in the laboratory. This took place regularly between the 3rd and 12th month in agar stabs kept at room temperature. When subcultures were tested at intervals, the zone of inhibition was seen to become progressively narrower, and by the 5th–6th

Table 3. *Mutual inhibition of* Staphylococcus aureus *strains shown by using a sensitive technique*

+ + + = Complete inhibition extending beyond area of growth of active strain.
+ + = Complete inhibition within area of growth of active strain.
+ & ± = Restricted zones of partial inhibition.
– = No inhibition.

	Active strain		Passive strain												
	Phage group	D.I.	1	2	3	4	5	6	7	8	9	10	11	12	13
1	71	+S	–	–	+++	+++	++	++	++	+	+++	+++	+++	+++	++
2	71	+S	–	–	+++	++	+++	++	+++	±	++	+	+++	++	+
3	Unclassifiable	+H	–	–	–	–	–	–	–	–	–	–	–	–	–
4	Unclassifiable	+H	–	–	–	–	–	–	–	–	–	–	–	–	–
5	I	+H	–	–	–	–	–	–	–	–	–	–	–	–	–
6	I	+H	–	–	–	–	–	–	–	–	–	–	–	–	–
7	I	+H	–	–	–	–	–	–	–	–	–	–	–	–	–
8	III	–	–	–	–	–	–	–	–	–	–	–	–	–	–
9	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	II	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11	Unclassifiable	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12	Non-typable	–	–	–	–	–	–	–	–	–	–	–	–	–	–
13	III	–	–	–	–	–	–	–	–	–	–	–	–	–	–

month was often reduced to a fine crevasse between the growth of the staphylococcus and the corynebacterium. Loss of inhibitory power was not associated regularly with any other change in cultural characteristics: most strains retained their original ability to form pigment, coagulase, haemolysin and hyaluronidase. Most were penicillin resistant and egg-yolk negative on first isolation and these properties were unchanged. Table 4 shows the results of 885 inhibition tests on Type 71 staphylococcal strains at various times after isolation; strains giving zones 1 mm. or more in radius in the routine test were considered to be still DI +. The results include those of the initial test on 534 strains (most, but not all, being done within 3 months of isolation) together with repetitions carried out on unselected batches of cultures. There was a small decrease in the proportion of positive results between the 3rd and 6th months and a much greater decrease subsequently; only 1 of 70 tests carried out after 12 months was positive. Staphylococci other than members of Type 71 which produced sharp zones of inhibition showed a similar loss of activity on storage, and nearly all were DI − after 1 year.

Table 4. *Results of inhibition tests on cultures of* Staphylococcus aureus *Type* 71 *stored in the laboratory for different periods of time*

	Months of storage				
	<3	3–6	6–9	9–12	>12
Number tested	481	211	76	47	70
Number inhibitory	450	180	44	2	1
% inhibitory	94	85	58	4	1

The ability to produce hazy zones of inhibition appeared to be unaffected by prolonged storage in the laboratory. Twenty-nine staphylococcal strains re-tested 12–21 months after isolation were active and gave zones as large as those seen when they were first tested. A culture of *Staphylococcus aureus* (NCTC 6507), which was described by A. Fleming as an inhibitor of *Corynebacterium diphtheriae* when deposited in the National Collection of Type Cultures in 1943 produced a good zone of hazy inhibition in 1955.

The nature of this loss of activity was investigated by a method which makes use of the fact that single colonies of a DI + staphylococcus deposited on a lawn of *Corynebacterium diphtheriae* of somewhat decreased thickness give rise to recognizable zones of inhibition. Loopfuls of a broth culture of a staphylococcus so diluted as to yield 20–30 colonies each were deposited on a fresh lawn of *C. diphtheriae* made by flooding a blood agar plate with a broth emulsion about 1/10 of the strength of that used in the routine inhibition test. When Type 71 staphylococcal strains, which gave somewhat narrowed zones of inhibition in the routine test were treated in this manner, two sorts of colonies were seen: larger, well-developed colonies surrounded by clear-cut zones of inhibition about 0·5 mm. in radius, and rather smaller colonies with no zone around them (Pl. 1, fig. 4). On subculture to blood agar the two sorts of colony were indistinguishable; the difference on the original plates was undoubtedly due to crowding of the non-inhibiting colonies by the corynebacteria. When the routine test was carried out on these subcultures, those from inhibiting colonies gave sharp zones of full width and those from non-inhibiting colonies were inactive. Repetition of the tests over a period of some weeks gave identical results, but when the selected variants were stored for some months the inhibition zones of the DI+ strains again became narrower and the cultures were again found to consist of mixtures of DI+ and DI− variants. Repeated examination of individual colonies from artificially selected DI− variants did not reveal the presence of any DI+ forms.

Changes in susceptibility to typing phages associated with loss of inhibitory activity

When strains of *Staphylococcus aureus* Type 71, which on first isolation had been lysed only by phage 71 at RTD, were re-typed some months later it was found that many of them had also become susceptible to phage 55 at RTD. This appeared to occur after about the same period of storage as the loss of ability to inhibit *Corynebacterium diphtheriae*. The association between the two changes was therefore investigated in 79 originally DI+ members of *S. aureus* Type 71 which had been isolated 5–30 months previously (Table 5). Sixty-two of the 65 strains which had become DI− were now lysed by phage 55; 57 of them reacted strongly. All but 1 of the 14 strains still DI+ were insusceptible to phage 55. The one exception was, in fact, an example of a more far-reaching change in phage pattern, as it was now also lysed by phages 3A, 3B and 3C. There was, therefore, a general tendency for the two changes to be associated, but exceptions occurred. However, it was also observed that 3 other members of Type 71 that were DI− on first isolation, also became fully susceptible to phage 55 within 1 year.

Table 5. *Acquisition of sensitivity to phage* 55 *by* Staphylococcus aureus *Type* 71 *strains, and its relation to loss of ability to inhibit* Corynebacterium diphtheriae

DI+ = zone of inhibition > 1 mm. radius.
Lysis by phage 55 = ++ = > 49 plaques; + = 20–49 plaques; ± = 1–19 plaques.

	Lysis by phage 55							
	DI−				DI+			
Period of storage	++	+	±	−	++	+	±	−
> 12 months	39	3	2	1	0	0	0	1
5–12 months	18	0	0	2	1*	0	0	12

* Now susceptible to phages 3A/3B/3C/55/71

A small number of staphylococcal strains which failed to react with phage 71 at RTD but were lysed by phage 71 (and usually by other Group II phages) at 1000 × RTD were included in Type 71 by Parker, Tomlinson & Williams (1955). Twelve of these so-called 'weak 71' strains, which were DI+ on first isolation, were re-tested after periods exceeding 1 year, by which time they had all become DI−. Eight now reacted with phage 71 at RTD, 1 with phage 55 and 1 with both 55 and 71: two were unchanged. Ten '71-like' strains from impetigo lesions, originally completely non-typable at RTD and at 1000 × RTD, but all DI + S, were also re-examined over one year after isolation. All were now DI – : two were lysed by both phages 55 and 71, 4 by 71, 3 by 55 and one by Group I phages, all at RTD.

It appeared, therefore, that no one phage pattern was exclusively associated with the presence or absence of inhibitory activity. Whatever the phage pattern of the original DI + S form, loss of inhibitory power was associated with a gain of susceptibility to Group II phages, and susceptibility to phage 71 usually but not always 'preceded' susceptibility to phage 55 in the course of dissociation.

Some of the discrepancies in the results shown in Table 5 might have been due to the fact that some of the staphylococcal cultures were in fact mixtures of DI + and DI – forms. Therefore, a number of freshly-selected DI + and DI – variants were obtained from cultures which gave narrow zones in the routine inhibition test, using the technique described in the previous section, and were re-typed. Table 6 shows the susceptibility to phage 55 of 118 such variants from 12 cultures. The general association of susceptibility to lysis by phage 55 with absence of inhibitory power was again seen: with most strains the difference was quite clear cut, but discrepancies occurred in a minority of strains.

Table 6. *Lysis by phage* 55 *of artificially-selected* DI + *and* DI – *variants from twelve* Staphylococcus aureus *Type* 71 *cultures*

Number of variants lysed to the degree shown.

	Degree of lysis by phage 55							
	DI – variants				DI + variants			
Staphylococcal strain no.	++	+	±	–	++	+	±	–
1	5	.	.	.	.	.	.	5
2	5	.	.	.	.	.	.	5
3	5	.	.	.	.	.	.	5
4	5	.	.	.	.	.	.	5
5	5	.	.	.	.	.	.	5
6	5	.	.	.	.	.	.	5
7	5	.	.	.	.	.	.	3
8	2	.	.	.	.	.	.	1
9	4	.	1	.	.	.	.	5
10	10	.	.	.	1*	.	.	9
11	2	.	.	.	1	1	2	1
12	5	.	.	.	2	2	.	1
	58	0	1	0	4	3	2	50

* Susceptible to phages 3A/3B/3C/55/71

Rountree (1956) attributed the resistance of *Staphylococcus aureus* strains to certain typing phages to the presence of symbiotic phage; this suggested that the step-wise gain in susceptibility to certain Group II phages which accompanies loss of inhibitory power might also be due to the loss of a phage. An attempt was therefore made to obtain a phage from the DI + form with which to lysogenize the DI – form. The two were grown together under a variety of conditions, but no such phage could be detected.

Attempts to induce lysis in mixtures by ultraviolet irradiation were also unsuccessful. Variants that were DI – and susceptible to phage 55 were successfully lysogenized with this phage and so rendered resistant to its action; but they remained DI –.

Among the artificially-selected variants shown in Table 6 was one with a grossly aberrant phage pattern (strong lysis by phages 3A, 3B, 3C, 55 and 71) which had retained its inhibitory activity. This appeared to be of some significance, since a whole culture which underwent the same change (71 to 3A/3B/3C/55/71) while remaining DI+S had been seen previously (Table 5), and also since a DI+S strain with the pattern 3A/3B/3C/55/71 had once been isolated from a case of impetigo. The artificially-selected variant 3A/3B/3C/55/71:DI+S had been found in a culture together with both the 71:DI+S and the 55/71:DI – forms, and when either of these forms was grown in broth the supernatant fluid contained a phage lytic for it. Phages derived from both forms were identical in their lytic spectrum on the standard propagating strains (strong lysis of PS 3C and weak lysis of PS 3A). Strong preparations of both phages were made by growth on 3A/3B/3C/55/71, and were spotted on lawns of the same strain. Subcultures from the resistant growth were made, and from them a strain lysed only by phage 71, but also DI+S, was obtained. The 3A/3B/3C/55/71 variant did, therefore, appear to have arisen from a loss of symbiotic phage; but the change was not accompanied by any alteration in the ability to inhibit corynebacteria.

Naturally-occurring staphylococcal cultures with the phage-pattern 55/71

The regular appearance of staphylococcal variants with the phage pattern 55/71 in laboratory cultures originally lysed only by phage 71 stimulated interest in naturally-occurring strains with the same pattern. The 55/71 variants had the same cultural characters as their parent Type 71 strains except for the negative DI reaction, i.e. they were egg-yolk negative and usually penicillin resistant. Nearly all naturally-occurring members of phage Group II, other than Type 71, are egg-yolk positive and usually penicillin sensitive. Further, Type 71 strains very seldom give rise to deep suppuration, while other members of phage Group II frequently do. In the series of 2032 cultures summarized in Table 1 there were 236 members of phage Group II other than Type 71, and 39 of them reacted only with phages 55 and 71. The origin and cultural characters of these 55/71 strains are shown in Table 7. Fifteen of them, including 6 from deep infections, resembled 'the rest' of phage Group II in being egg-yolk positive and DI –: all were penicillin sensitive. The other 24, all from impetigo or other vesicular skin lesions, were egg-yolk negative, all but one were penicillin resistant, but less than half were DI+. The DI – members of this latter group were, in fact, indistinguishable from DI – variants arising from Type 71 cultures *in vitro*. Such strains were encountered on several occasions in outbreaks of *pemphigus neonatorum* in which lesions of closely-related cases had yielded ordinary Type 71 strains. This had led to some difficulty in interpreting the typing results, but we had concluded that the strains were epidemiologically connected. An opportunity to investigate this situation further arose in connexion with a severe case of *pemphigus neonatorum* in a child born at home. Four cultures were obtained, one each from the infant's skin and nose, and one from each nostril of the midwife. All were typed and tested for inhibition of *Corynebacterium diphtheriae* within 1 week of isolation. The baby's skin strain was 55/71:DI− and his nose strain 71:DI+S. Of the two strains from the midwife, one was 55/71:DI− and the other, although thought to have been obtained from a single-colony 'pick' was a mixture of 55/71:DI− and 71:DI+S. It must be assumed, therefore, that the population in both the patient and the nurse was a mixed one.

Table 7. *Origin and cultural characteristics of* 39 *naturally-occurring staphylococcal strains with phage-pattern* 55/71

	Egg-yolk reaction			
	+		−	
Origin of strains	**DI+**	**DI−**	**DI+**	**DI−**
Impetigo	0	5	6	2
Pemphigus neonatorum	0	0	4	12
Deep suppuration	0	6	0	0
Normal nose	0	4	0	0

DISCUSSION

The observations recorded in this paper were made incidentally during an enquiry into the epidemiology of superficial skin infections. Many earlier workers had studied the inhibition of corynebacteria and other Gram-positive organisms by strains of *Staphylococcus aureus*, but their interest had been concentrated mainly on the mechanism of the inhibition, and on the part it might play in clearing the upper respiratory tract of *Corynebacterium diphtheriae*. The ability to inhibit appeared to be distributed in a haphazard fashion among strains of staphylococci and there was little indication that it might be a characteristic of a certain sort of staphylococcus. It was clear, however, that different staphylococci produced a variety of inhibitory agents, and that the number and type revealed would depend on the precise method used to detect them. It was fortunate that the simple method adopted by Tomlinson in his early work on impetigo should have been so well adapted for the detection of the agent produced by *S. aureus* Type 71. Members of phage Type 71 and a small number of related strains, all of which were associated with superficial vesicular skin lesions, produced a characteristic, narrow, sharply-defined zone. A further group of staphylococci produced an easily distinguishable zone with a hazy periphery: these strains had little else in common, except their lack of association with staphylococcal infections in man.

In recent years a number of bacterial species and serotypes have been split up into epidemiologically valid subdivisions by determining the range of activity of the inhibitory substances produced by individual strains. Fredericq, Betz-Bareau & Nicolle (1956) and Shannon (1957) produced typing systems for certain O-groups of *Escherichia coli*, Abbott & Shannon (1958) were able to divide *Shigella sonnei* into 7 colicine types and Thibaut & Fredericq (1956) further subdivided a group of *Corynebacterium diphtheriae* strains insusceptible to typing phages. In general, however, the members of the colicine or bacteriocine types so defined do not appear to differ from each other except in their ability to produce inhibitory agents, though Thibaut & Fredericq (1956) did observe that strains of *C. diphtheriae gravis* not producing agents active against certain indicator organisms were invariably fermenters of glycogen. So far, however, there has been no instance, other than that recorded here, of the production of a particular inhibitory agent only by strains with the ability to cause a particular sort of lesion.

Both the sharp and the hazy zones of inhibition of *Corynebacterium diphtheriae* appear to be due to agents produced by staphylococci during growth, which diffuse slowly through agar but not through cellophan, and which kill *C. diphtheriae*. Further investigation of the properties of these agents was hampered by our failure to obtain cell-free preparations of them. Others have found little difficulty in separating and concentrating the colicines (see Goebel, Barry, Jesaitis & Miller, 1955), the pyocines (Hamon, 1956) and the anti-staphylococcal agents produced by staphylococci (Halbert *et al.* 1953). However, Thibaut & Fredericq (1956) found that the agents produced by *C. diphtheriae* which inhibited other corynebacteria also diffused slowly in agar and could not be demonstrated in broth cultures.

There are several important differences between the agents responsible for sharp and for hazy zones of inhibition of *Corynebacterium diphtheriae*. Their range of activity on other corynebacteria is different: all or most of the staphylococci which inhibit the strain of *C. diphtheriae mitis* used in our routine test also inhibit a number of other corynebacteria, including other *mitis* and *gravis* strains and a number of diphtheroids, but a group of strains with recognizable colonial characters was found to be susceptible to inhibition by sharp zone forming but not by hazy zone forming staphylococci. Also, staphylococci which give sharp zones of inhibition of *C. diphtheriae* have a weak but definite inhibitory action on staphylococci not able to produce sharp zones, and there is some evidence that both activities are due to the same substance. On the other hand, strains forming hazy zones on *C. diphtheriae* had no action on other staphylococci.

Although the ability to produce hazy zones of inhibition persists indefinitely in culture, the ability to produce sharp zones is regularly lost after several months. This change takes place in one step and is associated with a broadening of susceptibility to lysis by typing phages. Staphylococcal strains at first lysed by phage 71 at RTD become susceptible also to phage 55, strains lysed by Group II phages only at 1000 × RTD become more susceptible to phage 71, or less often to phage 55, and strains previously untypable at 1000 × RTD become susceptible in many instances to phage 71, or to phage 55. This provides further evidence that certain 71-like strains are closely related to *Staphylococcus aureus* Type 71.

The authors are indebted to Miss Jean Perry of the Department of Medical Illustration, Crumpsall Hospital, Manchester, for the photographs shown in the Plate.

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EXPLANATION OF PLATE

Fig. 1. Inhibition of *Corynebacterium diphtheriae* by *Staphylococcus aureus*. Left: sharp zone; Right: hazy zone; × 1·5 Transmitted light.

Fig. 2. Sharp zone of inhibition. × 2. Reflected light.

Fig. 3. Hazy zone of inhibition. × 2. Reflected light.

Fig. 4. Single colonies of *S. aureus* from a stored culture on a lawn of *C. diphtheriae*. Inhibition zones around some colonies but not around others. × 4. Reflected light.

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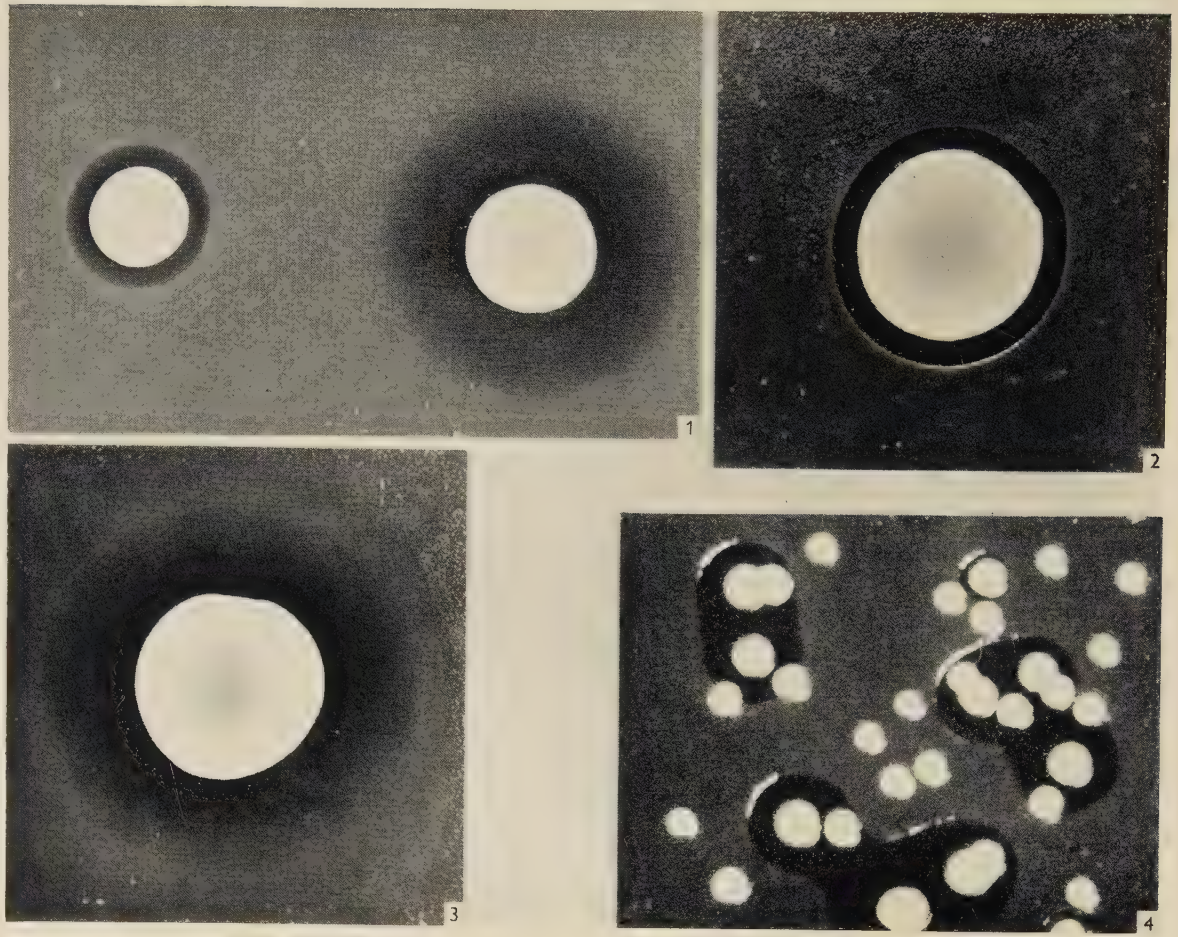

M. T. PARKER & L. E. SIMMONS—INHIBITION BY *STAPHYLOCOCCUS AUREUS*. PLATE 1

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Extracellular Polysaccharides of Rhizobium

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SUMMARY: The extracellular polysaccharides of seventeen strains (four species) of *Rhizobium* were studied chromatographically. The seven strains from lucerne (*R. meliloti*) produced polysaccharides which contained glucose but consistently lacked glucuronic acid. The remaining ten strains, which were isolated from pea (*R. leguminosarum*), clover (*R. trifolii*) and bean (*R. phaseoli*), produced a polysaccharide in which both glucose and glucuronic acid were demonstrated. These latter strains could be grouped into three types according to their glucose: glucuronic acid ratio, but such groups did not correspond with the species.

In 1931 Hopkins, Peterson & Fred identified glucuronic acid in the extracellular polysaccharide of strains of *Rhizobium meliloti*, *R. leguminosarum* and *R. trifolii*. Schluchterer & Stacey (1945) found that the polysaccharide from a clover strain consisted of a glucose-glucuronic acid chain. Contrary to these findings, De Leizaola & Dedonder (1955) found rhamnose, glucose, galactose, mannose, xylose and arabinose in preparations from four species of *Rhizobium* (pea, soya bean, clover, lupin), but reported complete absence of glucuronic acid. Kleczkowski & Kleczkowski (1952) reported having found fructose in a clover strain, as well as glucose and glucuronic acid in this and in a strain of *R. leguminosarum*. By using antisera developed against the parent bacterium, these workers obtained precipitation of antibody with the polysaccharide prepared from *R. leguminosarum*. With *R. trifolii* the polysaccharide did not cause precipitation in the homologous antiserum.

In the present investigation, polysaccharides from a selection of Rhizobium strains representing four species have been examined: (*a*) chromatographically, with a view to investigating the report of De Leizaola & Dedonder (1955); (*b*) quantitatively, by methods of spectrophotometric analysis, to determine their glucose: glucuronic acid ratio.

METHODS

Organisms and methods of culture. The following organisms were selected for study: *Rhizobium trifolii* (clover strains) SU36, SU46, SU64, SU91, SU298/533, NA30, NZ19; *R. leguminosarum* (pea strains) SU302, SU331; *R. phaseoli* (bean strains) NA1; *R. meliloti* (lucerne strains) SU27, SU47, SU62, SU74, SU76, SU101, SU277. (SU refers to strains from the Sydney University collection; NA, New South Wales Department of Agriculture; NZ, the Department of Scientific and Industrial Research, New Zealand.)

The organisms were inoculated into the medium formulated by Norris (1958) which contains (g./l.) mannitol, 10; disodium hydrogen phosphate, 0·45; magnesium sulphate heptahydrate, 0·1; potassium nitrate, 0·6; ferric chloride, 0·01; thiamin and biotin, trace. For each strain, four 1 l. flasks containing 100 ml. medium were inoculated and incubated at 26° for 14 days. The gum was prepared by centrifuging down the organisms at 800 *g*, and pouring the supernatant fluid, usually viscous and opalescent, into three volumes of ice-cold ethanol. With copious gum-producers strings of gum formed immediately; with other strains, the gelatinous precipitate of gum appeared only on standing overnight at 4°. The initial precipitate was centrifuged down, redissolved in water, again centrifuged to remove debris, and the supernatant fluid precipitated with three volumes of ethanol, usually slightly acidified with hydrochloric acid to coagulate the gelatinous precipitate. This twice-precipitated material, washed with ether and dried *in vacuo*, was used for most analyses. 'Gum' in this paper refers to these relatively crude polysaccharide (total carbohydrate) preparations which still contained protein and ash.

Hydrolysis of gum for chromatography. Gum (10 mg.) and 0·5 ml. N-HCL were sealed in an ampoule and heated at 100° for 20 hr. The hydrochloric acid was removed *in vacuo* over potassium hydroxide pellets and sulphuric acid, and the residue dissolved in an appropriate volume of water.

Chromatography. Descending paper chromatography was used with butanol + acetic acid + water (4 + 1 + 5) and butanol + ethanol + water (5 + 1 + 4) as solvents. *p*-Anisidine hydrochloride was used as developer for carbohydrates, and ninhydrin for amino acids. All identifications of spots were based on comparisons with authentic sugar samples.

Glucuronic acid analysis. This was done according to Bowness (1957) except that the 0·1 % (w/v) carbazole reagent recommended by Dische (1947) was used instead of the 0·2 % carbazole reagent used by Bowness. The more dilute reagent gave a lower blank reading and more consistent results. Analysis for glucuronic acid was carried out on samples (containing 250 μg. gum) of the solution of unhydrolysed gum used also for glucose analysis. Replicates were precise to ± 5 %. Standard curves for glucuronic acid (10–50 μg; Light & Co.) and glucose (50–200 μg.) were made with each batch of analyses. Absorption by glucose at 530 mμ was less than 10 % that of glucuronic acid. Before calculating the amount of glucuronic acid present, absorption by the gum solution was corrected for the amount of glucose found by anthrone analysis. Glucuronic acid added to a gum solution was 100 % recoverable. Glucose and glucuronic acid gave additive absorption. Ashwell (1957) has stated that protein below 0·1 % (w/v) does not interfere with the carbazole reaction.

Glucose analysis. It was not found possible to obtain reproducible results for glucose analysis by the carbazole method of Bowness (1957). The anthrone method of Seifter, Dayton, Novic & Muntwyler (1950) was used on solutions of unhydrolysed gum. Replicates precise to ± 1 % were obtained on samples containing 100 μg. gum. A standard curve for glucose (50–150 μg./5 ml. solution) and for glucuronic acid (10–50 μg./5 ml. solution) was made with each batch of analyses. Glucuronic acid always gave an absorption of less than 5 % that of glucose at 620 mμ; when necessary a correction for the glucuronic acid content of the gum was made.

Nitrogen analysis. Digestion by sulphuric acid and peroxide was followed by nesslerization.

Deproteinization. A protein fraction could be removed from the twice-precipitated gum by the deproteinization technique of Sevag, Lackman & Smollens (1935). It was also found that drying the gum *in vacuo* rendered part of it insoluble in water. This insoluble fraction did not contain carbohydrate and was probably denatured protein.

Spectrophotometry. A Hilger Uvispek was used throughout.

RESULTS

Yield of gums and constituent sugars

The strains of Rhizobium used differed in the quantity of gum produced; the results with chromatography are also shown in Table 1. In addition to the sugars identified in Table 1, all hydrolysates showed a small slow-moving spot. thought to be a di- or oligo-saccharide, possibly the cellobiuronic acid unit described by Schluchterer & Stacey (1945). A fast-moving substance (R_{glucose} 2·0–2·5 in butanol + acetic acid + water), which developed a bright pink coloration with *p*-anisidine hydrochloride in the cold, appeared with gum from all strains of *Rhizobium trifolii* except NA30. This substance was not xylose, as would be expected if it arose from decarboxylation of glucuronic

Table 1. *Amount of twice-precipitated gum produced by different strains of Rhizobium, and constituent sugars detected by chromatography*

		Presence of	
Organism	Yield (mg./l.)	Glucose	Glucurono-lactone, glucuronic acid
R. trifolii			
SU36	422	+	+
SU46	198	+	+
SU64	118	+	+
SU91	140	+	+
SU298/533	390	+	+
NA30	305	+	+
NZ19	714	+	+
R. leguminosarum			
SU302	770	+	+
SU331	215	+	+
R. phaseoli			
NA1	335	+	+
R. meliloti			
SU27	198	+	−
SU47	82	+	−
SU62	163	+	−
SU74	855	+	−
SU76	108	+	−
SU101	38	+	−
SU277	344	+	−

acid during hydrolysis. Its spot does in fact correspond with that of 4-*o*-methyl glucuronic acid from beech hemicellulose.

With ninhydrin developer, hydrolysates showed a similar pattern of eight amino acids (or groups of amino acids). These were still present after one cycle of deproteinization by Sevag's method. Amino acids were almost all absent from the gum of *Rhizobium trifolii* SU298/533 which had been separated from the water-insoluble fraction obtained after drying and then precipitated for a third time. Glucosamine was absent in all cases.

Solutions of the gums gave no coloration with iodine.

It was shown that replacement of mannitol in the culture medium by arabinose, glucose, xylose or sucrose as carbon source did not cause any alteration in the sugars present in the gum of *Rhizobium trifolii* SU298/533.

Table 2. *Quantitative analysis of Rhizobium gums*

Source of gum* and treatment	Composition of gum			Glucuronic acid in total carbohydrate (%)
	Nitrogen (%)	Total† carbohydrate (%)	Glucuronic acid‡ (%)	
R. trifolii				
SU36	—	41·8	9·8	23·5
SU46	—	56·3	13·7	24·4
SU64	—	54·7	13·7	25·0
SU91	—	52·3	13·3	25·4
SU298/533	3·8	47·2	11·8	25·0
NA30	2·7	50·4	15·2	30·2
NZ19	2·9	48·8	16·6	34·0
NZ19, from second washing of cells	1·7	53·2	17·8	33·4
NZ19, precipitated thrice	2·0	58·6	19·2	32·8
NZ19, insoluble matter removed, precipitated thrice	1·1	61·4	20·9	34·0
NZ19, Sevag-deproteinized once, precipitated thrice	1·0	62·2	20·2	32·5
R. leguminosarum				
SU302	2·7	48·8	7·6	15·6
SU331	—	53·2	16·2	30·5
R. phaseoli				
NA1	—	57·3	14·5	25·4
R. meliloti				
SU27	—	52·5	n.d.§	n.d.§
SU47	—	42·4	n.d.	n.d.
SU62	—	24·5	n.d.	n.d.
SU74	—	34·5	n.d.	n.d.
SU76	—	26·5	n.d.	n.d.
SU101	—	32·5	n.d.	n.d.
SU277	3·0	32·4	n.d.	n.d.

* All gums were precipitated twice from ethanol unless otherwise stated.

† Total carbohydrate = glucose + glucuronic acid.

‡ It seems probable that 4-*o*-methyl glucuronic acid would be included in this fraction.

§ n.d. = not detected.

Quantitative analysis of gums

The ash content of the gums varied between 4 and 12 %. About 12 % moisture which remained in the gum after drying *in vacuo* was removed by heating the gum at 100° for 2 hr. Ash, moisture, protein (nitrogen) and polysaccharide analyses accounted for up to 90 % of the weight of the gums.

The nitrogen content of the gums decreased on reprecipitation with ethanol and the content of polysaccharide increased (Table 2). However, the ratio glucose: glucuronic acid remained constant during purification, and also from one batch of culture to the next; this ratio therefore appears to be characteristic of the strain grown under these conditions.

In confirmation of the chromatographic observations, analysis showed that glucuronic acid, if present at all in the gums of the three lucerne strains, was below the limit of detection. The absorption curve of these gums in the presence of carbazole + sulphuric acid showed a very slight maximum at 530 mμ, which may have been due to a trace of glucuronic acid (less than 0·1 % of the gum).

Serology

We were not able to obtain convincing evidence of precipitation of a gum from either *Rhizobium trifolii* SU 298/533 or from *R. leguminosarum* SU 331 with the homologous antisera developed against whole organisms of the same strains. Some doubtful positives were obtained at relatively high concentrations of antigen and antiserum.

DISCUSSION

Cooper & Preston (1937) reported greater yields of polysaccharides from agar than from liquid medium (using a lucerne extract + minerals + carbon source mixture). This was not the case in the present study. Preliminary experiments, in which the organisms were grown on agar covered with cellophan (Bray, Schluchterer & Stacey, 1944), showed lower gum production than that obtained on Norris's defined liquid medium. Ash values for gums produced on Norris's medium were significantly lower than those found by Bray *et al.* (1944). This may be due to the absence of calcium salts from Norris's medium, since calcium is thought to combine with glucuronic acid in these polysaccharides (Cooper, Daker & Stacey, 1938). A fully defined medium, as used in the present study, eliminates the introduction of extraneous carbohydrate.

De Leizaola & Dedonder (1955) grew their organisms in a medium of mineral salts and glucose, with and without yeast autolysate; the gums studied chromatographically were those grown in the absence of the yeast autolysate. It is possible that the complete absence of glucuronic acid which these workers found with the pea, soya bean, clover and lupin strains was due to the absence of co-enzymes necessary for the production of glucuronic acid. The other sugars detected by these workers (rhamnose, xylose, arabinose, galactose, mannose) might conceivably have resulted from this disorganization of the mechanism for gum production, or from contamination of the gum preparations with cell walls and contents. Sparse practical details make it impossible to exclude the latter explanation. We did not detect fructose in any of the gums we studied (compare Kleczkowski & Kleczkowski, 1952).

In the present study only the lucerne organisms deviated from the 'classical' picture of Rhizobium gum (Hopkins *et al.* 1931) in their lack of glucuronic acid. Strominger, Maxwell, Axelrod & Kalckar (1957) described a uridine diphosphoglucose dehydrogenase in liver which, in the presence of co-enzyme I, produced uridine diphosphoglucuronic acid from uridine diphosphoglucose. This they postulated as a step in the synthesis of glucuronides and of polysaccharides containing glucuronic acid. Strominger & Mapson (1957) described a similar enzyme in pea seedlings. Smith, Mills & Harper (1957) compared capsulated and non-capsulated strains of Pneumococcus in an attempt to correlate uridine diphosphoglucose metabolism with ability to form a capsule. They concluded that failure occurred at some later stage than in the provision of building blocks. It is possible that the failure of *Rhizobium meliloti* strains to produce gums containing glucuronic acid may lie in the absence of such an enzyme system. This has not yet been demonstrated in Rhizobium, however.

Previous quantitative analyses on Rhizobium gums have all been performed by methods of carbon dioxide liberation which require at least five hundred times the quantity of polysaccharide used in our spectrophotometric method. Other spectrophotometric methods for glucuronic acid estimation (e.g. Hanson, Mills & Williams, 1944) are not valid in the presence of glucose. Bowness (1957) used the carbazole method for the analysis of hyaluronic acid, heparin and other polysaccharides. Like the carbazole estimation of glucuronic acid, the anthrone estimation of glucose has the advantage that previous hydrolysis of the polysaccharide, with the dangers of destruction or incomplete hydrolysis, is not necessary.

Hopkins, Peterson & Fred (1930) found with a lucerne organism that the uronic acid was 4–7 % of the gum (calculated on an ash-free basis); in two clover strains it was 22–25 % and in a pea strain, 19–22 %. The fact that their strain of *Rhizobium meliloti* (Wisconsin 100) yielded so much less uronic acid than the clover and pea strains provides a measure of agreement with our own failure to find glucuronic acid in any of the seven strains of the same species. Cooper *et al.* (1938) found 23–25 % uronic acid in a clover strain of Rhizobium. The analyses reported in Table 2 of our paper put the polysaccharides into four groups: (i) about 33 % glucuronic acid in the total carbohydrate (*R. trifolii*, NA30 and NZ19; *R. leguminosarum*, SU331); (ii) about 25 % glucuronic acid in total carbohydrate (*R. trifolii*, SU36, 46, 64, 91 and 298/533; *R. phaseoli*, NA1); (iii) about 15 % glucuronic acid in total carbohydrate (*R. leguminosarum*, SU302); (iv) glucuronic acid not detected (*R. meliloti*, SU27, 47, 62, 74, 76, 101 and 277).

It will be seen from this grouping that though the glucose: glucuronic acid ratio appeared to be constant for the strain that was investigated in more detail (NZ19, Table 2), it cannot be considered characteristic of species as they are at present defined. In other words, apart from the distinctiveness of the *Rhizobium meliloti* strains, there was no obvious relationship between the glucose:glucuronic acid ratios and invasive specificity. Although gums which have the same glucose:glucuronic acid ratio cannot on this evidence be considered identical, those with different ratios can certainly be regarded as different. Yet it might still be possible for such gums to contain a group-characteristic structure not revealed on over-all analysis. It does not appear from our precipitation tests, nor from those of Kleczkowski & Kleczkowski (1952), that specific agglutinability has any constant relationship to the gum fraction. Moreover, the production of an apparently similar polysaccharide by five strains of *R. trifolii* (SU36, SU46, SU64, SU91 and SU298/533) is not reflected in their somatic cross agglutinability. Strains SU36 and SU64 share an antigen, as do SU46 and SU91, but there is no cross-agglutination between the two pairs; nor does SU298/533 cross react with the other strains. Similarly the seven strains of *R. meliloti* include some which share a common agglutinogen and others which do not. The loss of gum in variants of SU298 (as well as SU297) does not prevent agglutination with antisera developed against the gummy types (Vincent, 1954) and *vice versa* (Bloomfield, private communication).

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A Transmissible Interfering Component of Vesicular Stomatitis Virus Preparations

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SUMMARY: Serial (more than two) undiluted passages of vesicular stomatitis virus in chick embryo cell monolayers gave low virus yields (e.g. 10 plaque-forming units (pfu)/cell) compared with dilute passage (e.g. 1000 pfu/cell). It was concluded that the low yields resulted from production of a transmissible interfering component since a mixture of undiluted passage with dilute passage virus stocks also gave low yields. Lack of infection of some cells, lack of time for complete growth, genotypic viral change, virus inactivation, poor cultures or media variations were excluded as explanations. Yield increase with inoculum dilution was marked with undiluted passage but not with dilute passage stocks. Multiple infection with live virus (dilute passage stocks) gave high yields, and mixed infection between plaque-forming units and transmissible interfering component (T) was required for interference and for propagation of T. Heat or u.v.-inactivation of live virus did not render it able to interfere (i.e. did not form T). The interference was mostly exclusion (all-or-none) with some slowing of release, since cells which released plaque-forming units released the normal number. T by itself was not toxic, and those cells which received T but not plaque-forming units in the first cycle of growth were protected from degeneration for several days. Cells which received T and plaque-forming units were killed whether or not they released virus.

Routine preparation of vesicular stomatitis virus stocks from chick embryo cell monolayers (Cooper, 1957*a*) sometimes gave apparent virus yields of less than 10 plaque-forming units (pfu)/cell (assuming that all cells in the monolayer released virus), while at other times the apparent yield was 1000 pfu/cell or more. A cytopathic effect was always marked or severe in all cells, when sufficient inoculum virus or time of growth was allowed for all cells to complete their virus release before harvesting. Poor culture conditions, media variations or inactivation of extracellular virus did not account for the low yields.

An incomplete form of the virus might have been responsible, since undiluted or low-dilution tissue-culture fluids were often used as inocula, although one or two undiluted passages usually gave high yields, and yields were in general unaffected by inoculum dilution (Cooper, 1958*a*). However, these experiments all used high-yield dilute passage stocks, since all low-yield stocks were rejected for experimental purposes. A study of the derivation of both Indiana and New Jersey serotype stocks revealed that low-yield preparations always arose by several serial low-dilution passages of initially high yield dilute passage stocks; this has now been frequently repeated. The present paper demonstrates that vesicular stomatitis virus, although not regarded as a myxovirus, may yet develop a serially transmissible but non-plaque-forming component which interferes with homologous virus growth; despite some analogies with the incomplete form of influenza virus, however, it cannot yet be regarded as an incomplete form of vesicular stomatitis virus. This is discussed by Bellett & Cooper (1959).

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METHODS

Preparation and storage of virus stocks and assays were as described by Cooper (1957*a*, 1958*a*). All assays and virus growth were made in 9 cm. diam. Petri dish monolayers containing $2 \pm 0{\cdot}5 \times 10^7$ chick embryo cells. Unless otherwise specified, the media used were as previously described except that the Earle's saline (ES) + 5 % (v/v) horse serum used for cell and virus growth was supplemented with 5 mg. lactalbumin hydrolysate/ml. and 1 mg. Difco yeast extract/ml. This supplemented Earle's saline is meant where 'medium' is subsequently mentioned without qualification. Adsorption of virus by monolayers for virus growth (but not assays) was carried out in the incubator hood described by Cooper (1958*b*); cells were resuspended for assay of infective centres as described in the same paper. Stocks are numbered according to serotype and type of passage, e.g. NJ.UP. 6, IND.DP. 25.

RESULTS

Effect of serial dilute and undiluted passage on virus yield/cell

Table 1 gives an abstract from the derivation of some fifty virus stocks of Indiana serotype used in experiments described here and elsewhere (Cooper, 1958*a*, *b*) and made in London in 1956–7. About twenty-five New Jersey stocks made at the same time, and twelve made in California in 1954–5 and used by Cooper (1957*a*, *b*, *c*; 1958*a*), had a similar derivation. Despite considerable individual variations in yield, one or two undiluted passages from a high-titre stock rarely gave low titres (particularly when of very dilute passage origin), but serial passage undiluted or at low dilution markedly lowered titres. Titres became high again on dilute passage.

Serial undiluted passage depressed New Jersey more than Indiana titres; one Indiana (Pl. 2, fig. 4) and three New Jersey undiluted passages showed less or no cytopathic effect after prolonged incubation despite the presence of adequate numbers of plaque-forming units. The controls were healthy microscopically, and simultaneous undiluted passage with dilute passage stocks gave good cytopathic effects. The titres were still those to be expected from unadsorbed inoculum, despite considerable thermal inactivation (3 days at 37°). The illustration of cell protection by a low-titred Indiana undiluted passage stock in Pl. 2, fig. 4, is compared with uninfected cells (Pl. 1, figs. 1, 2) and cells infected with an Indiana dilute passage stock (Pl. 2, fig. 3).

Unlike New Jersey virus, complete loss of Indiana virus cytopathic effect was not found on serial undiluted passage. Instead, undiluted passage yields stabilized at about 10 % of the dilute passage yields with occasional increases suggesting reversion (e.g. UP. 40, Table 1). One stock (IND.UP. 15) gave a

Table 1. *Abstract of genealogy of vesicular stomatitis virus stocks* (*Indiana serotype*)

Prepared by 30 min. adsorption of 0·5 ml. inocula by monolayers containing $2 \pm 0{\cdot}5 \times 10^7$ cells and overlaid by 5 ml. of medium. All monolayers inoculated on one date are comparable. The media were removed after 22 hr. and frozen for virus assay. Cytopathic effect was always very marked or complete while simultaneous controls were healthy. UP = undiluted passage, DP = dilute passage, the number being the stock number and the fraction being the dilution factor. The number in brackets = harvested yield in pfu/cell; '1 pfu' indicates stock prepared from a single picked plaque. 'u.v. treated' = u.v. irradiated to 0·17 % survivors, 'heated' (10 min. at 50°) to 2·1 % survivors, then both were plated undiluted with sufficient live UP. 22 virus (3 %, v/v) to infect all cells in the first cycle.

UP. 15 (12)
1 pfu
DP. 12 (800)
UP. 22 (550)

Date on which culture was inoculated		10^{-2}		10^{-3}	passed at 41°	u.v. treated		heated	
						10^{-3}		10^{-3}	
16. i. 57	UP. 26 (500)	DP. 17 (800)			UP. 34 (14)				
17. i. 57			UP. 25 (500)	DP. 16 (1060)		DP. 22 (1500)	UP. 43 (960)	DP. 21 (1250)	UP. 28 (1200)
22. i. 57	UP. 30 (15)		UP. 28 (50)	UP. 29 (400)	UP. 35 (275)	UP. 42 (500)	UP. 44 (75)	UP. 41 (110)	UP. 39 (40)
23. i. 57	UP. 32 (50)		UP. 31 (175)		UP. 36 (250)		UP. 45 (300)		UP. 40 (250)
24. i. 57	UP. 33 (125)				UP. 37 (250)				

high-titre undiluted passage yield and the same yield on dilute passage (Table 4) strongly suggesting reversion; it was grown at 32°, but this experiment was not repeated.

When the dilute passage and undiluted passage stock titres are grouped without considering their parentage in more detail, the mean titres in pfu/ml. are: New Jersey, $1{\cdot}7 \times 10^9$ (dilute passage) $1{\cdot}3 \times 10^8$ (undiluted passage); Indiana, $2{\cdot}7 \times 10^9$ (dilute passage) and 6×10^8 (undiluted passage). Thus mean undiluted passage and dilute passage titres differed significantly; since many 'dilute' passages were at low dilution, often from an undiluted passage parent, and some undiluted passage stocks were directly derived from high-titre dilute passage stocks, this significance is enhanced by the need for several serial undiluted passages before the yield was greatly lowered. In fact, if there were an equal number of alternating dilute and undiluted passages one might see no difference.

Factors other than interference which may contribute to a low yield

The existence of interference is postulated later; meanwhile the preceding data, which show low yields with some inocula but not with others, are not significant without considering the system used.

Culture variations. The culture method used (Cooper, 1957*a*) usually gave a nearly confluent monolayer with cells partly spread after 20 hr. (Pl. 1, fig. 1), becoming fully confluent by 46 hr. (Pl. 1, fig. 2) by cell spreading and enlargement rather than by cell division (Cooper, 1957*b*). The amount of debris was minimal; $1\text{–}2 \times 10^7$ cells were resuspended by trypsin and the microscopical appearance of the cultures was very reproducible. Some non-confluent batches, or individual 'toxic' monolayers, were discarded. Except with very dilute virus inocula, the cytopathic effect at 20–24 hr. after inoculation was always extensive in all cells whether with undiluted or dilute inocula, undiluted or dilute passage stocks or with high or low yields (Pl. 2, fig. 3). Four exceptions to a complete cytopathic effect have been mentioned, and were due to interference. Table 1 compares dilute and undiluted passages in single batches of monolayers, and shows the dependence of yield on the history of the stock. Growth curve studies (Cooper, 1957*a*, *b*, *c*; 1958*a*) showed that monolayers of one batch released virus very similarly, although some batch-to-batch variation occurred. It is concluded, therefore, that variations in the cultures used did not account for the low yields obtained with certain inocula.

Effect of different media. Apparently identical well-spread monolayers were formed in the medium generally used or in medium '199' (Morgan, Morton & Parker, 1950) + 5% (v/v) rabbit or horse serum or chick embryo extract. Any of these media, used for monolayer formation or virus release, allowed undiluted passage yields characteristic of stock history and independent of medium.

Inactivation of extracellular virus. The heat lability of vesicular stomatitis virus (half-life 1–2 hr. at 37° for both serotypes; Fig. 1) will decrease the apparent yields between release and harvesting, but when undiluted passage and dilute passage stocks were compared with the same opportunities for thermal inactivation (same multiplicity of infection) the yields were still low and high, respectively. There was no evidence for an increased heat lability on serial undiluted passage, as a permanent change is excluded by obtaining high yields again on one dilute passage (single plaque). Figure 1 shows no marked phenotypic difference between the stabilities of dilute passage and undiluted passage infective components.

Interference by an accumulation of thermally inactivated virus in the stocks

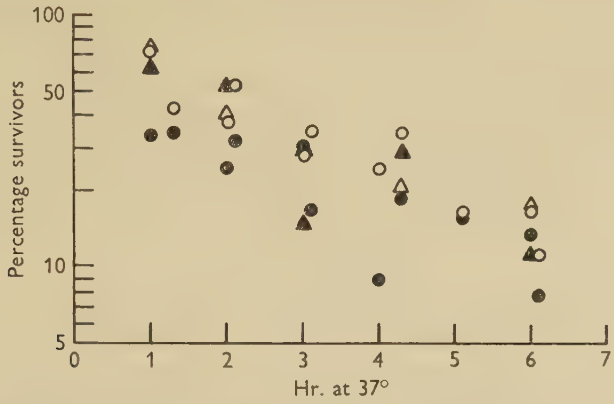

Fig. 1. The stability of vesicular stomatitis virus in medium (supplemented Earle's saline plus 15 % (v/v) heat-inactivated horse serum) at 37°. Stocks were diluted to about 500 pfu/ml. in warm medium, and samples were taken at intervals and assayed at once for infectivity. The T-containing stocks IND.DP. 6 (●) and NJ.UP. 7 (▲) were compared with relatively T-free stocks IND.UP. 22 (○) and NJ.DP. 10 (△).

Table 2. *The stability of vesicular stomatitis virus in buffer at various pH values and in other media*

The buffer solutions (M-citrate in M-phosphate) were adjusted to various pH values with a pH meter and N-HCl, and autoclaved. A small amount (0·1 ml.) of a New Jersey serotype virus stock of known titre was added to 4 ml. medium to give $1{\cdot}5 \times 10^6$ pfu/ml., and the suspensions were stored at 0° for 21 hr. when they were assayed at once.

Medium	Infectivity remaining after 21 hr. at 0°. (Percentage of original)
Buffer pH 9·1	33
8·0	27
7·1	29
5·8	23
4·9	11
4·1	3·5
3·0	*c.* 0·1
2·0	< 0·1
0·25 M-sucrose	3·9
0·88 M-sucrose	< 0·1
Distilled water	29
Earle's saline	49

is not an adequate explanation, since washing the cells after infection during serial undiluted passage did not increase the yield; virus killed by heat, storage at $-20°$ or u.v. irradiation does not interfere homotypically (Cooper, 1958*b*) and did not depress yield when added deliberately (Table 4). Small changes in pH value or osmotic pressure did not appear to affect infectivity, since the rate of loss of infectivity by vesicular stomatitis virus is the same between pH 6 and 9, although much higher below pH 5 (Table 2); the half-life was similar in distilled water, buffer or Earle's saline (about 10 hr. at 0°), although much less in sucrose solutions. Undiluted passage gave low yields from undiluted passage stocks and high yields from dilute passage stocks whether or not the serum had previously been heated at 56° for 30 min. No antibodies fixing complement with undiluted virus stocks were found by Brooksby's (1948) method in the horse serum used, although complement fixation was very good with specific guinea-pig immune sera. Thus, low yields from some inocula but not others cannot be accounted for by extracellular inactivation of virus.

Increasing yield with increasing inoculum dilution with undiluted but not dilute passage stocks

In a number of experiments, simultaneous comparison of undiluted and dilute passage yields with the same undiluted passage stock and batch of monolayers showed that dilute inocula gave higher yields than the same preparation inoculated undiluted, similar to the effect described by von Magnus (1951) for influenza virus. No difference in yield between undiluted passage or a wide range of dilute passages of New Jersey dilute passage stocks of high titre had previously been found (Cooper, 1957*a*, 1958*a*). This suggested that, like influenza virus, the effect of inoculum dilution on yield is more marked with undiluted than with dilute passage stocks. Tables 3–6 confirm the difference between such stocks in the effect on yield of inoculum dilution for both vesicular stomatitis serotypes. Each table represents one experiment with one batch of monolayers; subsequent assays were also performed with a single batch of monolayers.

IND.UP. 22 as dilute passage stock (high titre, one undiluted passage from a high titred dilute passage stock and giving high titres on further undiluted passage) was compared with IND.DP. 6 as undiluted passage stock (low titre, from a long undiluted + low dilution passage series, and giving low titres on further undiluted passage (Tables 3 and 4)). The paradox in nomenclature emphasizes the importance of serial rather than a single undiluted passage (for IND.UP. 22) in inducing this interference, and the inefficacy of one low-dilution passage (for IND.DP. 6) in removing it. IND.UP. 22 showed perhaps a little, while IND.DP. 6 showed a marked, decrease in yield at the two highest concentrations.

The lack of dilution effect with dilute passage New Jersey stocks is shown in Table 5. Table 6 compares two stocks of an undiluted passage series, NJ.DP. 8 (of high titre and giving a fairly high titre on one undiluted passage but derived by one very low dilution (1:2) passage from the original stock) and NJ.UP. 12 (of fairly high titre but giving very low titres on subsequent undiluted passage and derived from NJ.DP. 8 by one undiluted passage). Both NJ. DP. 8 and NJ.UP. 12 gave marked dilution effects, but the effect with the later undiluted passage stock was greater than with the earlier, especially at the highest concentrations.

Table 3. *The effect of inoculum dilution on 24 hr. yields from a serial low-dilution passage Indiana serotype vesicular stomatitis virus stock* (*IND.DP*.6, $8{\cdot}6 \times 10^{7}$ pfu/ml.) *using monolayers of one batch*

The 0·5 ml. inocula were removed after 1 hr. in the incubator hood, and 5 ml. of warm medium were added for virus release at 37°. M = input multiplicity of infection; cytopathic effect was severe in all cells at harvest

Inoculum dilution	M	Yield pfu/cell
Undiluted	2	8
1/2	1	82
1/8	0·25	315
1/16	0·12	177
1/32	0·06	248
1/320	0·006	200

Table 4. *The slight effect of inoculum dilution on 20 hr. yield from certain Indiana serotype vesicular stomatitis virus stocks*

The following stocks were compared: a dilute passage stock after one undiluted passage (IND. UP. 22); a serial undiluted passage stock which has reverted to non-auto interference (IND.UP. 15); IND.UP. 22 stocks containing a large excess of either ultraviolet- or heat-inactivated Indiana virus. For the latter, some IND.UP. 22 stock was u.v. irradiated to 0·17 % survivors, and some more heated to 2·1 % survivors (10 min. at 50°); sufficient untreated IND.UP. 22 was re-added (3 %, v/v) to infect all cells in the first cycle on undiluted passage. The inocula (0·5 ml.) were removed from monolayers after 1 hr. in the incubator hood, which were washed once with PBS and then given 5 ml. of warm medium for virus release at 37°; all monolayers showed complete or severe cytopathic effect at 20 hr. M = multiplicity of infection of live virus; the multiplicity of u.v.- and heat-inactivated virus will be the same as that of live IND.UP. 22.

	IND.UP. 22 (2×10^{9} pfu/ml.)		u.v. treated IND.UP. 22 (7×10^{7} pfu/ml.)		Heated IND.UP. 22 ($1{\cdot}1 \times 10^{8}$ pfu/ml.)		IND.UP 15 (5×10^{7} pfu/ml.)	
Inoculum dilution	M	Yield pfu/cell	M	Yield pfu/cell	M	Yield pfu/cell	M	Yield pfu/cell
Undiluted	50	500	2	960	2·5	1200	1·2	>500
1/2	25	660	1	670	1·2	1500	0·6	1500
1/4	12	840	0·5	1000	0·6	1250	0·3	900
1/10	5	1250	0·2	1110	0·25	610	0·12	1250
1/20	2·5	1000	0·1	1000	0·12	1250	0·06	960
1/100	0·5	1250	0·02	1500	0·025	1050	0·012	1000
1/1000	0·05	1060	0·002	1500	0·0025	1250	0·0012	1000

The yield on undiluted passage of dilute passage stocks in presence of u.v.- and heat-inactivated virus, and at 41°

Addition of u.v.- and heat-inactivated dilute passage stocks to the same untreated dilute passage stock (Table 4) did not cause any depression of yield on one undiluted passage, and further undiluted passage (Table 1) did not suggest that much interfering component had been produced on this passage.

Table 1 shows that, whereas one undiluted passage at 41° gave a low yield, further undiluted passage of this low yield stock gave high yields indicating that there had been no accumulation of the interfering component during growth at the higher temperature. It would seem therefore that the interfering component differs fundamentally from heat- or u.v.-inactivated virus.

Table 5. *Lack of effect of dilution on* 10 *hr. yield of a dilute passage New Jersey serotype vesicular stomatitis virus stock* ($9{\cdot}2 \times 10^8$ *pfu/ml.*)

Inocula (0·5 ml.) were adsorbed for 30 min., the monolayers washed twice with medium and overlaid with 5 ml. warm medium for virus release at 37°.

Inoculum dilution	Multiplicity of infection	10 hr. yield (pfu/cell)
Undiluted	23	180
1/2	12	220
1/5	4·5	200
1/10	2·3	220
1/20	1·2	180
1/50	0·45	200
1/100	0·23	220
1/200	0·12	115

Table 6. *The effect of inoculum dilution on* 22 *hr. yields from two serial low-dilution New Jersey serotype vesicular stomatitis virus stocks, compared using one batch of monolayers*

NJ.UP. 12 was derived from NJ.DP. 8 by one undiluted passage. The 0·5 ml. inocula were removed after 1 hr. in the incubator hood, monolayers were washed once with 5 ml. PBS, and 5 ml. warm medium added for virus release at 37°. M = input multiplicity of infection of live virus. Cytopathic effect was severe in all cells at harvest.

	NJ.DP. 8 ($2{\cdot}7 \times 10^9$ pfu/ml.)		NJ.UP. 12 (1×10^9 pfu/ml.)	
Inoculum dilution	M	Yield (pfu/cell)	M	Yield (pfu/ml.)
Undiluted	60	11	25	0·90
1/3	20	*c.* 20	8	*c.* 0·5
1/10	6	*c.* 30	2·5	*c.* 15
1/30	2	65	0·8	50
1/100	0·6	55	0·25	*c.* 20
1/300	—	—	0·08	40
1/1,000	0·06	65	0·025	*c.* 5
1/10,000	0·006	180	0·0025	185
1/100,000	0·0006	100	0·00025	90

Comparative one-step virus release from monolayers infected with dilute or undiluted passage stocks

A comparison of one-step virus release was made between (*a*) cells completely infected (multiplicity = 6) with dilute passage stocks, and (*b*) cells with the same multiplicity of infective virus but also with a high multiplicity of the interfering component T, which is discussed below.

Figure 2 and Table 7 show one of three such experiments. A high proportion (about 80 %) of the cells which had received infective virus had nevertheless been prevented from releasing infective progeny, so that infective virus was presumed to be 'excluded' by some component of the undiluted passage stock. The other experiments, summarized in Table 8, show a similar degree of exclusion, and in all three experiments those cells able to release virus did so to an approximately normal yield (84–178 % of controls), as the total yield of the culture was reduced by about the same factor as the number of cells able to release virus. Thus the lowered yield of the undiluted passage

Table 7. *Prevention of virus release by the T component*

Of a batch of 20 monolayers of $1{\cdot}5 \times 10^7$ cells, one group of 10 received a dilute passage stock as inoculum (IND.DP. 18, $2{\cdot}7 \times 10^9$ pfu/ml. diluted 1/10 in medium to give a multiplicity of infection of 10), and the other group of 10 received an undiluted passage stock (IND.DP. 18 diluted 1/10 in IND.UP. 11, $< 5 \times 10^7$ pfu/ml., giving an infective multiplicity = 10 but with additionally an excess of T). After 1 hr. adsorption at 37°, while still in the incubator hood the inoculum was removed, cells washed once with warm PBS, and 5 ml. warm medium were added to each monolayer. After 3 hr. infection, cells from 2 monolayers of each group were trypsinized into 1 ml. PBS for infective centre assays; the table refers to the composition of this suspension. The supernatant medium was removed from the remaining monolayers, still at 37°, at intervals for virus assay during 1-step growth (see Fig. 2). A and B (col. 1) are duplicates.

	Infective centres per monolayer					
Monolayer (1)	No. recovered $\times 10^6$ (2)	Not sedimented by 3 min. at 1000 rev./min. (free virus) (3)	Total cells recovered per monolayer $\times 10^7$ (4)	Plating efficiency $= \frac{\text{col } 2 \times 100}{\text{col. } 4}$ (5)		Probable number of cells releasing virus per monolayer (corrected for plating efficiency) $= \frac{\text{col. } 5 \times 1{\cdot}5 \times 10^7}{10{\cdot}1}$ (6)
Dilute passage inoculum A	2·2	—	1·6	13·8	10·1	$1{\cdot}5 \times 10^7$
Dilute passage inoculum B	0·84	6×10^3	1·3	6·5		
Undiluted passage inoculum A	0·20	—	1·4	1·4	2·25	$3{\cdot}3 \times 10^6$
Undiluted passage inoculum B	0·31	2×10^3	1·0	3·1		

Table 8. *Comparison of the plating efficiencies of the infected cell suspensions with final yields during one-step growth of virus from dilute passage (DP) and undiluted passage (UP) stocks*

Figure 2 and Table 7 describe expt. 3, and expts. 1 and 2 are duplicates of 3. Plating efficiency = infective centres/ml. divided by total cells/ml.

Expt. no. (1)	Plating efficiencies of infected cell suspensions 1 hr. after infection (ratio UP:DP) (2)	Time of harvest of final yield (hr. after infection) (3)	Final yields in total pfu/culture (ratio UP:DP) (4)	Yields per yielding cell (ratio UP yield:DP yield = col. 4/col. 2) (5)
1	0·200	10	0·168	0·84
2	0·105	24	0·166	1·6
3	0·218	24	0·39	1·8

stock is accounted for almost entirely by this exclusion, so that the excluding component is also the yield-reducing component. The latent period of the yielding cells in the interfered cultures was normal in length, although the rate of release was somewhat slowed. Despite the complete prevention of release from most of the cells, cytopathic effect was severe in all cells at 22 hr. as in other undiluted passage cultures.

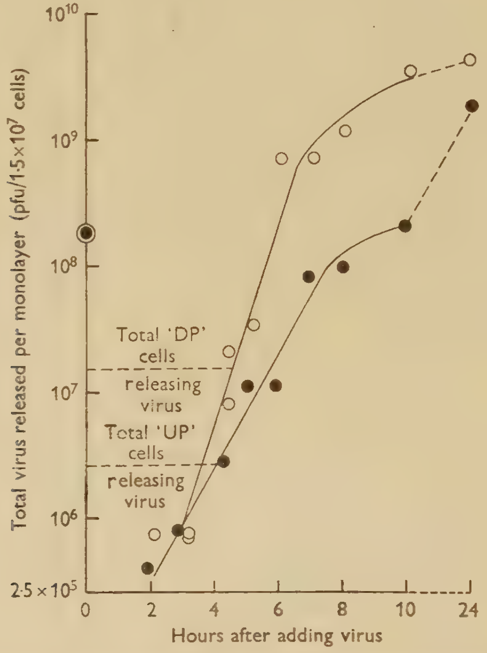

Fig. 2. The effect of the T component on one-step vesicular stomatitis virus release from identical chick embryo cell monolayers. Half of the monolayers received dilute passage virus sufficient to infect all the cells (○), the remainder received the same multiplicity of dilute passage virus with additionally a high multiplicity of T (●); full experimental details are given in Table 7. The supernatant fluid was removed from one monolayer of each group after various intervals at 37° and frozen pending assay of pfu. The point on the ordinate represents infective virus initially added, and the broken horizontal lines the number of cells/ml. able to release virus, corrected for plating efficiency (see Table 7) to give a truer estimate of latent period.

DISCUSSION

As compared with serial dilute passage, serial undiluted passage of dilute passage vesicular stomatitis virus stocks of either serotype (New Jersey or Indiana) gave low yields of infective virus, unaccounted for by variations in media, cultures or heat labilities. As a single dilute passage of the low-yielding stock, sometimes from a single plaque, gave high yields again, there must have been no genotypic change in the majority of the virus particles. Multiple adsorption of infective virus alone (dilute passage inocula) did not give low yields, but yields increased with dilution of undiluted passage stocks. In some cultures apparent protection of nearly all cells through many viral growth cycles, with no evidence of cytopathic effect and very low yields, resulted from low-titre undiluted passage stocks containing insufficient infective virus to infect all cells initially. A mixture of dilute and undiluted passage stocks infected far less cells than the same dilute passage stock alone. We can only conclude that undiluted passage vesicular stomatitis virus stocks contain a large proportion of a non-plaque-forming component which can interfere with virus growth or release. There is much less in dilute passage seeds, and it is self-duplicating or transmissible in the sense that it stimulates its own formation by infective virus and is not diluted out on successive passage but tends to increase in amount.

The analogy with the familiar 'incomplete' form of influenza virus seems very strong. However, because another type of interfering component exists ('interferon', Isaacs & Lindenmann, 1957), and because this interfering substance of vesicular stomatitis virus is not yet demonstrably 'virus' in serology and size, it will be called the transmissible interfering component, 'T'. Some relations between T, incomplete influenza virus and interferon are discussed by Bellett & Cooper (1959).

The way in which T is made remains unknown. Ultraviolet, 'storage' (−20°) and thermal (37° and 50°) extracellular inactivation of infective vesicular stomatitis virus does not create T; nor does the inactivated virus produce much T when allowed to react with cells; the one passage at 41° (IND.UP. 34, Table 2) suggests that thermal intracellular inactivation gives similar results. Also the high yields on further undiluted passage of this stock and that stock made at 32° (IND.UP. 15) suggests that T synthesis is slower than infective virus synthesis at temperatures away from the optimum. T is made only slowly, if at all, in cells not containing infective virus, as otherwise one could not obtain T-free stocks by passage sufficiently diluted to make first-cycle mixed infection between T and infective virus unlikely, but insufficiently diluted to separate them entirely by end-dilution. This also suggests that T is not simply a non-cytopathogenic variant of the virus. Placing infective virus on all cells (thereby insuring at least some first-cycle mixed infection between T and infective virus), on the other hand, clearly allows T to be reproduced well. Only a minority of such cells liberates infective virus; which group liberates T remains unknown.

Regarding the way in which T reduces the yield of a culture, those cells yielding virus release a normal number of plaque-forming units although the release rate may be slower. Very many 'infected' cells cannot release infective virus at all, and exclusion in this sense accounts for nearly all the depression of yield. The widespread cytopathic effect shows that all cells were indeed 'infected', and that the infection, however aborted, is lethal. Exclusion is also shown by the protection of all cells in the three New Jersey low-dilution passages where less than 1 pfu was released per cell, and where cytopathic effect was absent. These indicate that T is not toxic by itself, and suggest that cell breakdown may not occur if enough time is allowed between adsorption of T and of infective virus.

Thus interference by T may take three forms: (*a*) a slowing of release of infective virus, (*b*) 'exclusion' (no release) with cell degeneration, (*c*) exclusion with no cell degeneration. These may conceivably reflect differences in time and quantities of adsorption of T and plaque-forming units, or different forms of T. The delayed degeneration in Pl. 2, fig. 4 arose with a multiplicity of infective virus of 0·3–1, where for probably half the cells at least 4 hr. elapsed between receiving T and infective virus. Cytopathic effect was less delayed for diluted (10^{-3}) T-free stocks, or for T-containing stocks of higher plaque-forming titre where a much shorter interval would elapse between adsorption of T and of infective virus.

It is interesting that ultraviolet, heat or 'storage' inactivated (and probably live also) dilute passage vesicular stomatitis virus cannot rapidly exclude homotypically (Cooper, 1958*b*), yet T can. Among other viruses, one wonders in how many cases of interference by killed or live homologous virus the presence of undetected T-like components can be ruled out.

Because of many genetically 'incomplete' bacterial strains, Burnet (1955) felt that all viruses may exist in some sort of incomplete form, but in its original meaning (von Magnus, 1951) of a transmissible non-cytotoxic interference it is harder to find without a haemagglutinin; reproducible growth and assay systems helped the recognition of T in vesicular stomatitis, in which it is not very obvious. Nevertheless, T-like components may occur among animal viruses other than myxoviruses. Mims (1956) concluded from results similar to the above that the transmissible interfering component of Rift Valley fever was 'incomplete' virus. However, environmental factors complicate *in vivo* interpretations, and the interfering component may not be the one reacting with or producing specific antibodies, so that as with vesicular stomatitis virus direct evidence of incomplete virus is lacking. Chambers (1957) showed that persistence of western equine encephalomyelitis virus in strain 'L' cells for many subcultures was due to a transmissible interfering component protecting most cells from cytopathic effect. Dilute inocula caused more cytopathic effect than less dilute, but thermally inactivated virus was not ruled out as the protective agent. On subculture in antiserum, and occasionally spontaneously, virus was lost and the cells became susceptible again, so that lysogeny or genotypic cell resistance seem excluded. Analogous persistence in a continuously growing culture might also be expected with vesicular stomatitis virus because (*a*) T can protect most cells from virus, (*b*) some cells receiving T and virus can yet release progeny virus, (*c*) T can be serially transmitted for many passages. T-like components could well account for instances of persistence in other viruses.

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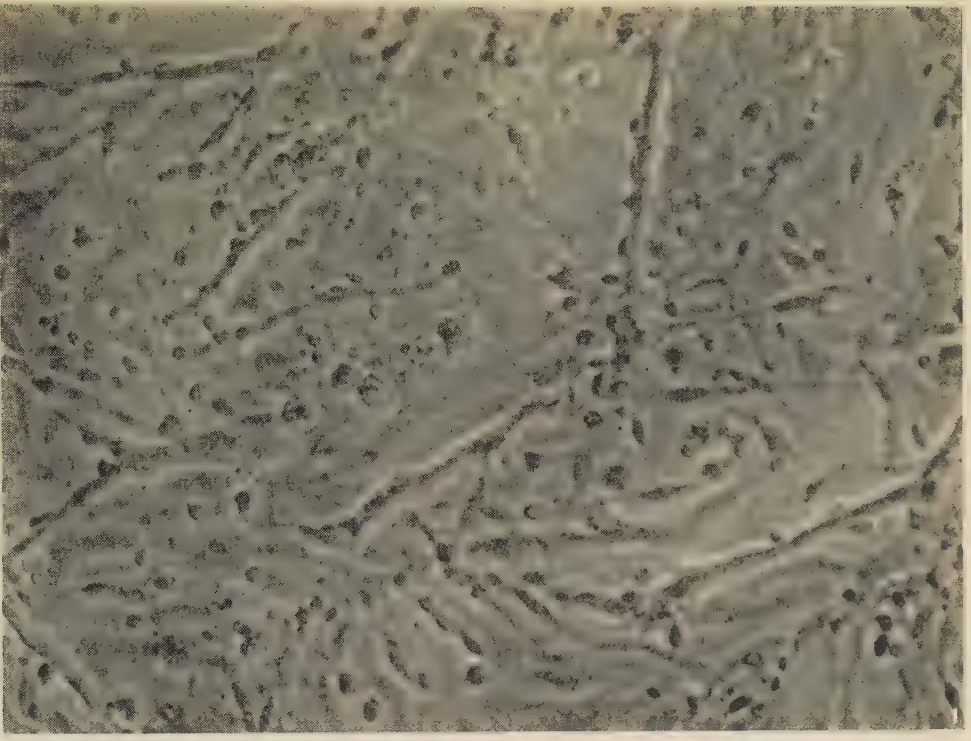

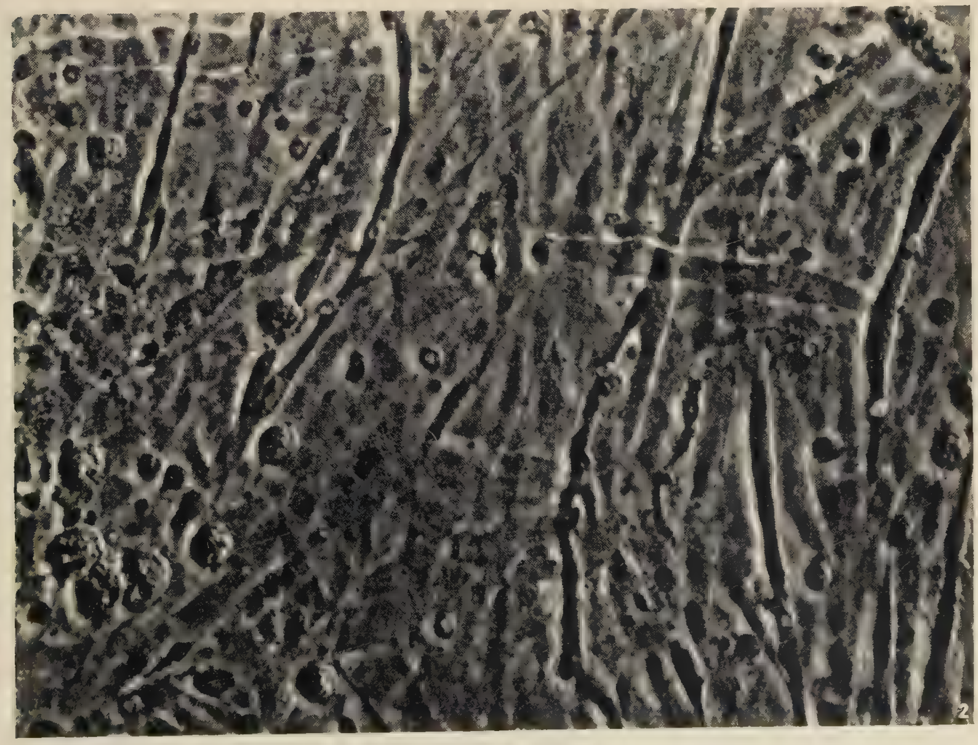

P. D. COOPER & A. J. D. BELLETT—TRANSMISSIBLE INTERFERING COMPONENT. PLATE 1

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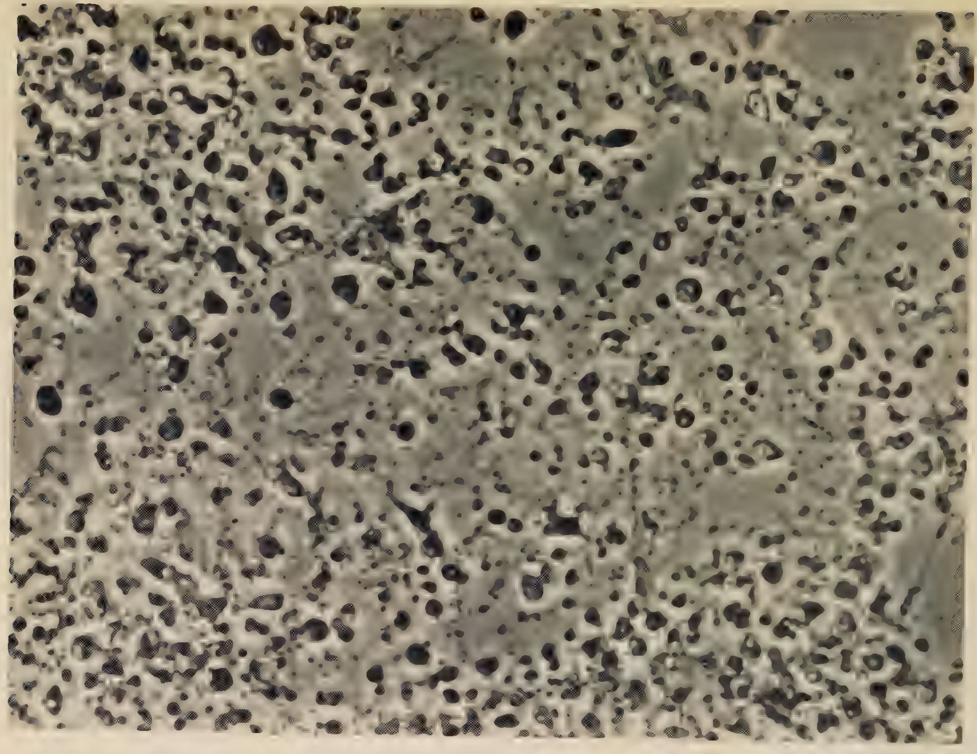

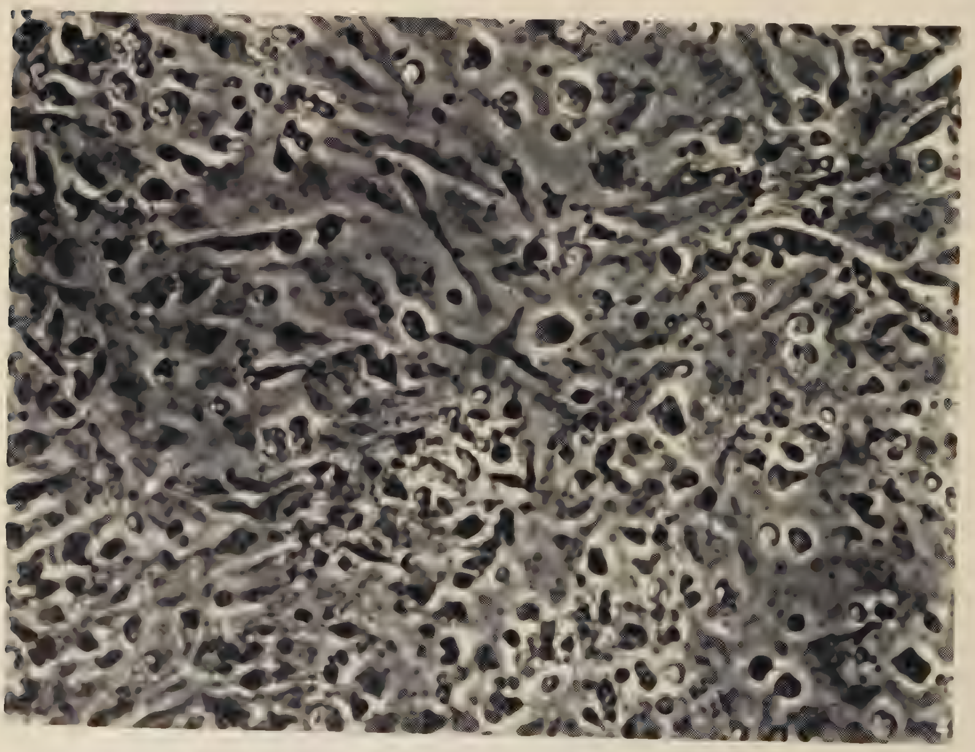

P. D. COOPER & A. J. D. BELLETT—TRANSMISSIBLE INTERFERING COMPONENT. PLATE 2

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EXPLANATION OF PLATES

Plates 1, 2, figs. 1–4 are phase-contrast pictures of living unstained chick embryo cell monolayers all of one routine batch, treated simultaneously in a manner identical except for type of inoculum. × 280.

Plate 1

Fig. 1. Typical 20 hr. monolayer at usual stage of inoculation with virus. Cells are partially spread and nearly confluent.

Fig. 2. Typical 46 hr. monolayer, uninoculated. Cells are very well spread and confluent.

Plate 2

Fig. 3. 46 hr. monolayer, 26 hr. after inoculation with 0·5 ml. IND. DP. 18 ($2 \cdot 7 \times 10^9$ pfu/ml.) and overlaying with 4·5 ml. medium. Cytopathic effect is maximal or very extensive in all cells, and many had resuspended from the glass. This effect is typical of most undiluted passage and all dilute passage stocks with inocula undiluted or diluted to 10^{-3}.

Fig. 4. 46 hr. monolayer, 26 hr. after inoculation with 0·5 ml. IND.UP. 11 (5×10^7 pfu/ml.). Most cells showed some cytopathic effect being either rounded or with granular cytoplasm but without the extensive degeneration of Fig. 3. The culture had degenerated by 120 hr.

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Some Properties of the Transmissible Interfering Component of Vesicular Stomatitis Virus Preparations

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SUMMARY: In vesicular stomatitis virus inocula containing the transmissible interfering component (T) an exponential relation, at low doses, between inoculum concentration and virus yield suggested that adsorption of one T particle was enough to exclude infective virus. This relation was not maintained at high doses of inoculum, possibly because maximal interference required several hours between adsorption of T and virus particles. An assay method for T, based on the dose which gave 37% of the yield from T-free inocula, showed that the T content of inocula was usually related to passage history and yield of virus on undiluted passage. Treatment with immune serum did not appear to neutralize T, which sedimented in the centrifuge more slowly than did virus. T was much less rapidly inactivated at 56° and by u.v. irradiation than was the infectivity. Despite resemblances between the agent T, incomplete influenza virus and interferon, direct evidence is lacking that T is an incomplete form of the vesicular stomatitis virus or an interferon-like substance.

In a previous paper (Cooper & Bellett, 1959) we described a phenomenon with vesicular stomatitis virus which resembled the interference due to 'incomplete' virus in influenza (von Magnus, 1951). It was concluded that the low yields from serial undiluted passage of virus were due to a transmissible interfering component (T). The present paper attempts a further definition of the properties of the agent T, and a comparison of T with incomplete influenza virus and influenza interferon (Isaacs & Lindenmann, 1957).

METHODS

The preparation of media, virus stocks and tissue cultures, and the assay of virus by plaque count were described previously (Cooper, 1957; Cooper, 1958*a*; Cooper & Bellett, 1959). Virus titres are expressed as plaque-forming units (pfu)/ml., dilutions being made in phosphate-buffered saline (PBS).

Assay of the interfering agent T. Samples (0·5 ml.) of the virus stock to be assayed in twofold serial dilutions in the medium composed of Earle's saline + 5 mg. lactalbumin hydrolysate/ml. + 2 mg. yeast extract/ml. + 5% (v/v) horse serum were added to washed confluent (20 hr.) chick embryo cell monolayers. After 1 hr. at 37° 5 ml. of 'conditioned' medium (fluid from 20 hr. monolayers) were added, and 20 hr. later the supernatant fluids were removed and plaque-assayed immediately, or after 2–4 days at −20°. The derivation of the content of T agent from these values is discussed below.

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Immune serum. Standardized hyperimmune guinea-pig sera prepared with the Indiana or New Jersey serotypes of vesicular stomatitis virus were kindly supplied by Dr J. B. Brooksby (Research Institute, Pirbright, Surrey). These sera in a final dilution of 1/300 prevented plaque formation by homotypic virus, but not by heterotypic virus.

Complement-fixation tests. Complement-fixation tests (Brooksby, 1952) determined colorimetrically the haemolysis of sensitized sheep erythrocytes by complement left unfixed by antigen + serum mixtures. A series of 1 ml. volumes of 1·25-fold dilutions of preserved guinea-pig serum (Wellcome Laboratories) in veronal buffer was incubated for 1 hr. with 0·2 ml. antigen and 0·4 ml. of 1/16 dilution of hyperimmune serum in colorimeter tubes in a water bath at 37°. Haemolytic serum (Wellcome Laboratories) diluted 1/100 was added to an equal volume of 3 % (v/v) sheep red blood cells and put in a water bath at 37° for 30 min. Two ml. of this sensitized red cell suspension were added with mixing to each colorimeter tube, and the tubes were left at 37° for a further 30 min. for haemolysis, then centrifuged at 2000 rev./min. for 5 min. and the colour of the supernatant immediately compared with distilled water in a Hilger–Spekker absorptiometer using a green filter. The points of 50 % haemolysis were obtained graphically using a probit plot and compared with a control complement titration to calculate the amount of complement fixed. Results (Table 6) are expressed in arbitrary units/ml., the units representing the difference between relative concentrations of complement required to give 50 % haemolysis in presence and absence of antigen (concentration in absence of antigen = 1).

High-speed centrifugation to separate T from infective virus. Samples of undiluted passage virus stocks (3 ml.) were added to 5 ml. capacity glass centrifuge tubes which each contained 1 ml. of solidified agar gel (20 g./l. distilled water), and centrifuged at 13,500–14,000 rev./min. for 65 min. in a high-speed angle head ('International' refrigerated centrifuge) at 5°. The top 2 ml. of the supernatant was withdrawn for assay of T, plaque-forming units and complement-fixing activity.

RESULTS

The number of T particles required to exclude virus

Cooper & Bellett (1959) concluded that the low yields from serial undiluted passage of vesicular stomatitis virus stocks were caused by a transmissible interfering component, T. This will be considered to be particulate, even though it may be of molecular dimensions. Since it was also shown that the interference was mostly an all-or-none exclusion, where cells either released a normal yield or none at all, the ratio of the overnight yield from a T-containing inoculum to that from a T-free control can be taken as approximately the proportion of cells which did not receive an effective dose of T. The T-free control can either be a dilute passage stock, or the T-containing stock diluted sufficiently to make first-cycle mixed infection between T and plaque-forming units negligible.

A plot of the relative concentration of dilutions of T added to a series of monolayers (the stocks being serially diluted down to doses at which further dilution did not increase the yield) against the logarithm of yield relative to the maximum yield (multiplicity of infective virus being kept constant at 2·5) showed a negative exponential relationship between dose and cells remaining uninterfered with, suggesting that only one T particle per cell was required to exclude virus (Fig. 1). Experiments with some other T-containing stocks (together with one which was free of T), similar to that of Fig. 1 except that multiplicity of infection was not constant, showed that this exponential relation applied generally at low, but very rarely at high, doses (Fig. 2). This is discussed in the next section.

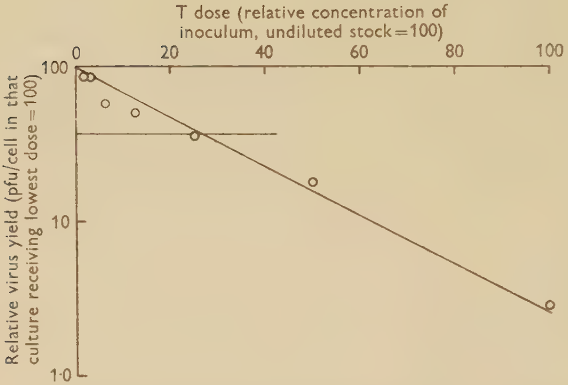

Fig. 1

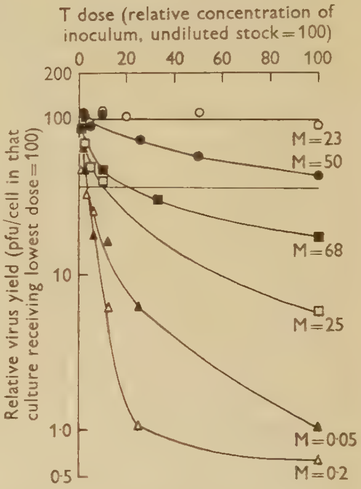

Fig. 2

Fig. 1. The effect of T concentration on virus yield with a constant multiplicity of infective virus. Mixtures of NJ.UP. 17 and NJ.DP.12 were prepared so that each mixture contained 10^8 pfu/ml., while the T content varied from $3{\cdot}2 \times 10^8$/ml. to $2{\cdot}7 \times 10^6$/ml. Inocula of 0·5 ml. of each mixture were added to 20 hr. monolayers, 5 ml. medium added after 1 hr. adsorption at 37°, and the supernatants plaque-assayed after a further 20 hr. at 37°. The highest multiplicity of T was 4 particles per cell, but the multiplicity of infective virus was always 2·5 pfu/cell. Relative yield is calculated on the basis of 2×10^7 cells/monolayer, although not all cells yield virus.

Fig. 2. The effect of dilution of inoculum on yield for a number of virus stocks. T and infective virus were thus both varied together. The values of M refer to the multiplicity of infective virus with undiluted stock as inoculum (dose = 100). Δ = NJ.UP.17, ▲ = NJ.UP.16*a*, □ = NJ.DP.12, ■ = NJ.DP.8, ● = IND.UP.22, ○ = NJ.DP., stock, not numbered. Relative yield is calculated on the basis of 2×10^7 cells/monolayer, although not all cells yield virus.

The assay of T

As mentioned above, the ratio between overnight yields from T-containing and T-free inocula can be taken as the proportion of cells which did not receive an effective dose of T, provided that enough time is allowed for virus release from all unprotected cells to be complete. When a culture has received an average of one effective dose of T/cell, the proportion of cells not receiving an effective dose of T, i.e. the proportion of yielders, should equal e^{-1}(= 0·37). Since 1 effective dose = 1 particle (Figs. 1, 2), the inoculum dilution giving a 37 % yield will contain about 4×10^7 T particles/ml., since 0·5 ml. is always added as inoculum to monolayers containing about 2×10^7 cells. From this the T content of the undiluted stock could be calculated; in those stocks with apparently low efficiencies of interference at high doses, the 37 % yield was estimated from the low-dosage part of the curve. It must be recognized that (like plaque-forming units) the 'T particle content' is defined only by the assay system used, and, in common with any other biological activity estimated in terms of particles, is likely to represent a minimum due to probability effects and other causes of inefficient assay.

The following section shows that several such assays give results in agreement with expectations from undiluted passage yield and passage history of the stock. However, most assays involved simple serial dilution of the stock, so that low doses of T accompanied low doses of virus, and a later section suggests that an interval of several hours between receiving T and plaque-forming units is needed for maximal interference. The reduced effectiveness of T at high doses may be because a small variable proportion of cells is insusceptible to T but susceptible to virus, or because T is antagonized by some component of the stock, but it is more likely to be because the higher doses infect more cells in the first cycle (i.e. within say 30 min. of T). If T requires more time than this to protect all cells, then those cells infected in the first cycle will be more likely to succumb to virus. In support of this are the two assays of T in NJ.UP.17 afforded by Figs. 1 and 2. In Fig. 1 all cells received plaque-forming units within a short time of T, and the apparent T content was $1 \cdot 5 \times 10^8$ T particles/ml.; in Fig. 2, at the dose giving 37 % yield probably less than 1 % of the cells received plaque-forming units within 4–5 hr. of T, and the apparent T content was $6 \cdot 4 \times 10^8$ T particles/ml.

Presumably this perturbation will not affect the T assay if the curves are linear in that segment where the input multiplicity of live virus is appreciably less than 1. However, several curves are non-linear in this segment (Figs. 2, 3), so that, unless T is still not fully established by the third cycle, some other factor may also neutralize T. Therefore, while this assay does permit some interpretations in terms of absolute T particle concentrations, one can summarize its defects as (*a*) it requires a lengthy series of plaque assays and several assumptions, (*b*) it is interfered with by infective virus, (*c*) its reproducibility may be further affected by some other unknown factor.

Relation between T content, yield on undiluted passage and passage history

This relationship is summarized for a number of stocks in Table 1. As may be expected from the data of Cooper & Bellett (1959), stocks giving high yields on undiluted passage generally had high plaque-forming unit and low T contents, and stocks giving low yields had the reverse. Consequently, high-yielding stocks often had low T:plaque-forming unit ratios and vice versa.

Table 1 may suggest that this ratio is more closely related to undiluted passage yield than are absolute T or plaque-forming unit contents, but the data are here insufficient to show whether infective virus could compete with T and so neutralize the interference.

Table 1 also shows that serial undiluted passage usually resulted in a high, and dilute passage or a single undiluted passage a low, T content. The T content of dilute passage stocks depended on the dilution of passage, as may be expected, but some stocks passed at quite high dilutions (e.g. NJ.DP. 12) had a relatively high T content.

Table 1. *Comparison of history of several vesicular stomatitis virus stocks with T content and autointerfering ability*

IND. = Indiana serotype, NJ. = New Jersey serotype, UP. = undiluted passage, DP. = dilute passage.

Stock	Yield on undiluted passage (pfu/cell)	T content (particles/ml.)	Infective titre (pfu/ml.)	T:pfu ratio	History
IND.UP.22	500	$4\cdot8\times10^7$	2×10^9	1:42	1 UP from one pfu
NJ.DP.14	375	$< 10^7$	$2\cdot6\times10^9$	1:>260	1 DP from one pfu
IND.DP.3	60, 72, 205	*c.* 10^9	$3\cdot2\times10^9$	1:3·2	1 UP+1 DP (1/3) from one pfu
NJ.DP.8	10, 250	$3\cdot1\times10^8$	$2\cdot7\times10^9$	1:8·8	1 DP (1/2) from original stock
IND.UP.13	70	$5\cdot6\times10^8$	$2\cdot4\times10^8$	2·3:1	Long UP+very low-dilution passage series from 1 pfu
NJ.UP.17 plus NJ.DP.12	55	$1\cdot5\times10^8$	1×10^8	1·5:1	Artificial mixture of NJ.UP. 17 plus NJ.DP. 12
IND.DP.24	22	$2\cdot5\times10^8$	$2\cdot1\times10^9$	1:8·4	1 UP.+3 very high-dilution passages from 1 pfu
NJ.DP.12	6	$2\cdot8\times10^8$	1×10^9	1:3·6	Long high-dilution passage series
NJ.UP.16*a*	2, 2	4×10^8, 8×10^8	$1\cdot9\times10^6$	210:1	Long UP. or low-dilution passage series from 1 pfu
NJ.UP.17	1·6	$6\cdot4\times10^8$	$7\cdot2\times10^6$	89:1	Long UP. or low-dilution passage series from 1 pfu

Time required for establishing exclusion by T

Although this time was not measured directly, some indications of its duration have been obtained. In one series of monolayers (Table 2) inoculated simultaneously with a constant amount of T and varying amounts of infective virus, those cultures with an average input multiplicity of infection considerably less than one gave a yield appreciably less than those with multiplicities around one, despite greater chances for thermal inactivation in the latter.

Thus, although interference was present in all cultures, those cells which were not infected until 4–6 hr. after receiving T (i.e. until the second cycle) were more resistant to the virus. The culture with the lowest inoculum showed some delay in cytopathic effect, a phenomenon never found with comparable inocula of dilute passage virus.

Table 2. *Effect on yield of varying the multiplicity of infective virus, T being kept constant*

A series of monolayers was infected with 0·5 ml. each of mixtures containing constant amounts of T but different amounts of plaque-forming units. After 45 min., 5 ml. of medium were added, and the supernatants harvested for plaque assay after a further 20 hr.

Inoculum per cell		Yield	Cytopathic effect at
pfu	T particles	(pfu/cell)	20 hr.
3·2	4·9	18	+ +
1·6	4·5	18	+ +
0·85	4·3	42	+ +
0·44	4·1	42	+ +
0·24	4·1	5·8	+ +
0·14	4·0	9·0	+ +
0·045	4·4	2·0	±

This protection was also found several times with undiluted passage virus where most cells were not infected in the first cycle (Cooper & Bellett, 1959). In experiments where T and plaque-forming units were added simultaneously to all cells, about 80 % of cells were prevented from releasing virus, but not protected from cytopathic effect. We therefore conclude that the bulk of the exclusion is established within 1 hr. but that longer, perhaps 6–8 hr., is required to protect cells fully (especially from cytopathic effect). These times may well depend on relative multiplicities of T and plaque-forming units; interference is the resultant of the two processes of establishing 'infection' by T and by infective virus, and as mentioned by Cooper (1958*b*), one cannot deduce much about the rates of the two component processes from the apparent rate of establishing interference.

Heterotypic interference by undiluted passage virus stocks

Three groups of monolayers received either (*a*) Indiana virus alone, or (*b*) Indiana virus plus a little New Jersey virus plus New Jersey T particles in excess, or (*c*) Indiana virus plus the same small addition of New Jersey virus as an extra control (Table 3). All monolayers had the same multiplicity of Indiana virus. As the infective New Jersey virus was present in minor amounts and is less effective than Indiana virus in establishing exclusion (Cooper, 1958*b*), its contribution to yield and infective centres was ignored; immune serum was not used. Table 3 shows that the presence of undiluted passage New Jersey virus considerably depressed both yield and infective centres formed by Indiana virus, in amounts not due to the small presence of New Jersey infective virus. The interference was only partly accounted for by the decrease in the number of infective centres established by the dilute passage Indiana virus. Thus, undiluted passage New Jersey virus has a heterotypic interfering activity not shown by dilute passage New Jersey virus at the same multiplicity, although the relationship between the heterotypic excluding agent and T remains to be demonstrated.

Table 3. *Heterotypic interference by undiluted passage virus stocks*

Three groups of identical 20 hr. monolayers received 0·5 ml. per monolayer of: *Group* 1. Indiana virus alone (1·5 ml. IND.DP. 24 1/50 (PBS) + 1·5ml. PBS, giving $2\cdot1 \times 10^{7}$ IND. pfu/ml.); *Group* 2. Indiana virus + New Jersey virus + New Jersey T particles (1·5 ml. IND. DP. 24 1/50 (PBS) + 1·5 ml. NJ.UP. 17, giving $2\cdot1 \times 10^{7}$ IND. pfu/ml. + $3\cdot6 \times 10^{6}$ NJ. pfu/ml. + $3\cdot2 \times 10^{8}$ NJ.T particles/ml.); *Group* 3. Indiana virus + T-free New Jersey virus in the same multiplicity of infection as group 2 (1·5 ml. IND.DP. 24 1/50 (PBS) + 1·5 ml. NJ.DP. 12 1/100 (PBS), giving $2\cdot1 \times 10^{7}$ IND. pfu/ml. + 5×10^{6} NJ pfu/ml.). After 30 min. at 37°, 2 monolayers in each group received 5 ml. of conditioned medium (harvested for virus assay 20 hr. later), and the remaining 2 monolayers were harvested for total cell and infective centre assay (Cooper, 1958*b*). M = multiplicity of infection.

Group (1)	Inocula (2)	Infective centres/ monolayer $\times 10^{5}$ (3)	Total cells/ monolayer $\times 10^{7}$ (4)	Mean* plating efficiency (5)	Total yield (pfu/ monolayer) $\times 10^{8}$ (6)	Mean† yield (pfu/ infected cell) (7)
1	IND virus (M = 0·5)	5·9 6·5	1·6 1·4	8·2	120 110	1580
2	IND virus (M = 0·5) + NJ virus (M = 0·09) + NJ T particles (M = 8)	1·9 3·0	1·3 1·5	3·4	2·4 15	280
3	IND virus (M = 0·5) + NJ virus (M = 0·11)	7·5 9·4	2·8 2·8	9·8	120 30	860

* Plating efficiency = ratio of infective centres expected from inoculum : infective centres found.

† Based on infective centre count corrected for plating efficiency in control (Group 1), i.e. col. 3 is multiplied by 100/8·2.

The failure of immune serum to neutralize heterotypic interference by undiluted passage virus stocks

Three confluent monolayers received either (*a*) Indiana virus + Indiana T particles, (*b*) Indiana virus + Indiana T particles + Indiana antiserum, or (*c*) medium alone, as primary inocula (Table 4). After adsorption they were challenged with New Jersey virus as secondary inocula, and the supernatant fluids assayed for New Jersey plaque-forming units after virus growth and release. Table 4 shows that the Indiana antiserum did not prevent the Indiana undiluted passage virus from interfering with growth of New Jersey virus.

Table 4. *The failure of immune serum to neutralize heterotypic interference by undiluted passage virus stocks*

One 20 hr. monolayer received 0·5 ml. IND.UP. 49 ($1\cdot7\times10^{8}$ pfu/ml., $4\cdot4\times10^{8}$ T/ml.), another received 0·5 ml. IND.UP. 49 plus 0·2 ml. Indiana antiserum (1/2 PBS), and a control monolayer received 0·5 ml. ES. After 30 min. at 37°, 0·5 ml. NJ.DP. 14 diluted 1/10 ES ($2\cdot6\times10^{8}$ pfu/ml.) was added to each monolayer as secondary inoculum and 5 ml. medium were added 30 min. later. The fluids were harvested and titrated 8 hr. after infection, Indiana antiserum (0·2 ml. of 1/10 dilution in PBS) being added to the agar overlayers.

Primary inoculum	Secondary inoculum	Yield (pfu/ml.)
0·5 ml. IND.UP. 49	0·5 ml. NJ.DP. 14 (10^{-1})	$5\cdot2\times10^{7}$
0·2 ml. IND. antiserum (1/2) +0·5 ml. IND.UP. 49	0·5 ml. NJ.DP. 14 (10^{-1})	$3\cdot4\times10^{6}$
0·5 ml. ES	0·5 ml. NJ.DP. 14 (10^{-1})	$2\cdot5\times10^{8}$

The effect of heat and ultraviolet treatment on homotypic interference by undiluted passage virus stocks

Vesicular stomatitis virus minimally inactivated by heat or ultraviolet light, and therefore probably live virus also, does not interfere with the multiplication of infective virus of the same serotype if added simultaneously (Cooper, 1958*b*). If an undiluted passage stock is treated with heat or u.v. it should therefore be possible to follow the effect of the treatment on the interfering ability of T without complication from interference by inactivated virus.

Table 5 describes an experiment in which a T-containing New Jersey stock was exposed to u.v. or heated at 56° for varying times. After treatment samples

Table 5. *Heat and ultraviolet treatment of undiluted passage virus stocks*

A stock of NJ.UP.16 virus ($9\cdot0\times10^{7}$ pfu/ml.) was divided into 1 ml. samples in 5 ml. screw-capped bottles. One bottle was removed to 4° in the dark as a control; some bottles were put in a 56° water bath and others, with their caps removed, were put 50 cm. below a Philips 30 W. u.v. lamp. All were frequently shaken. After the exposure times shown, samples were removed to 4° in the dark and then titrated by plaque count. Half (0·5 ml.) of each sample was added to a monolayer, with medium alone on another as control, and all were followed 10 min. later by 0·25 ml. NJ.DP.14 diluted 1/2 in medium (giving 16 pfu/cell), and 5 ml. medium after a further 30 min. at 37°. The yields were harvested and titrated 20 hr. later.

			Yield from 0·5 ml. samples + 0·25 ml. NJ.DP.14	
Treatment	Sample time (min.)	Residual virus (pfu/ml.)	pfu/ml.	% control
Control (medium only)	—	—	$1\cdot3\times10^{9}$	100·0
None	0	$9\cdot1\times10^{7}$	$1\cdot9\times10^{7}$	1·5
56°	5	$3\cdot5\times10^{6}$	$8\cdot6\times10^{7}$	6·6
56°	10	$6\cdot0\times10^{4}$	$1\cdot3\times10^{8}$	10·0
56°	15	$1\cdot6\times10^{5}$	$1\cdot8\times10^{8}$	13·9
56°	20	$2\cdot2\times10^{4}$	$2\cdot3\times10^{8}$	17·7
56°	25	$1\cdot0\times10^{4}$	$3\cdot6\times10^{8}$	27·7
u.v.	10	$2\cdot7\times10^{7}$	$4\cdot0\times10^{7}$	3·1
u.v.	20	$7\cdot3\times10^{6}$	$2\cdot4\times10^{7}$	1·8
u.v.	30	*c.* $2\cdot0\times10^{6}$	$3\cdot0\times10^{7}$	2·3

were at once compared with an untreated control for plaque-forming unit contents, and the infectivity was found to decrease approximately exponentially with time of treatment in both cases. The full series of assays required for quantitative T comparisons were not performed, but the interfering abilities were compared by their effect on the overnight yield from a standard New Jersey inoculum. Table 5 shows that no detectable effect on interference followed a u.v. dose decreasing the plaque-forming unit content to 2 % of the original. On the other hand, a large drop in infectivity at 56° (to 10^{-4} of the original) accompanied a progressive but not complete drop in interfering activity. We conclude that T is much more stable to u.v. and 56° inactivation than is the infectivity, but at doses equally lethal for infectivity a temperature of 56° is probably a more effective inactivator of T than is u.v.

Separation of T and plaque-forming units by centrifugation

Samples of clarified medium and disrupted cells from monolayers infected with a T-containing inoculum, harvested early (8 hr. after infection) to minimize contamination with 'soluble antigen', were centrifuged as described so as to sediment most but not all of the infectivity. The products were compared with uncentrifuged samples with respect to infectivity, T content and complement fixation (Table 6 and Fig. 3). It was hoped thus to separate T from plaque-forming units, and to indicate a method for separating T from the bulk of the complement-fixing activity in order to study the serological nature of T.

Table 6. *High speed centrifugation of an 8 hr. undiluted passage virus stock*

Four monolayers each received 0·5 ml. of NJ.UP. 16 ($9{\cdot}0 \times 10^7$ pfu/ml.) and 5 ml. of conditioned medium after 1 hr. at 37°. The medium was harvested 8 hr. after infection; the monolayers were washed twice with PBS, the cells resuspended with trypsin (1·25 mg./ml. in PBS without Ca^{++} or Mg^{++}), washed in PBS, pooled and suspended in 5 ml. PBS for disruption by twice freezing at −60° and thawing at 37°. Both medium and cell debris preparations were centrifuged at 3000 rev./min. for 15 min.; 3 ml. of each supernatant were then recentrifuged at *c.* 14,000 rev./min. for 65 min. in an International centrifuge at 5°, when the top 2 ml. were withdrawn and compared with the supernatant before high-speed centrifugation with respect to infectivity, T content and complement fixation. The arbitrary complement-fixation units are described under 'Methods'; antiserum or antigen alone fixed negligible complement. The assay method for T is described in the text, and the detailed results of the T assays (relationship between yields and seed dilutions) are given in Fig. 3.

Sample	Titre (pfu/ml.)	Units complement fixed/ml.	T particles/ ml.
Medium before centrifugation	$1{\cdot}9 \times 10^6$	16·0	$8{\cdot}0 \times 10^8$
Medium, supernatant from 65 min. at *c.* 14,000 rev./min.	$2{\cdot}0 \times 10^5$	12·3	$6{\cdot}6 \times 10^8$
Cell debris before centrifugation	$1{\cdot}0 \times 10^5$	76·2	Not assayed
Cell debris supernatant from 65 min. at *c.* 14,000 rev./min.	$1{\cdot}4 \times 10^4$	83·0	Not assayed

The cell debris had a high complement-fixing activity which was not deposited on centrifuging, and had a low plaque-forming activity 90 % of which was deposited. From the medium containing released virus *c*. 90% of the infectivity was also deposited, while the complement-fixing activity of the supernatant dropped by about 20%. The T content of the tissue culture fluid was also decreased by 20% on centrifuging.

It can be concluded that the interfering activity characteristic of T was present in the 8 hr. yield of undiluted passage virus and had a much smaller sedimentation constant than the infective virus. Little quantitative significance can be attached to the differences in complement fixing activity or T content before and after centrifuging the preparations; they indicate that T is not readily separated from virus antigen by centrifugation, precluding for the present any identification of T with virus serological activity because of probable contamination with soluble antigen (Bradish, Brooksby & Dillon, 1956).

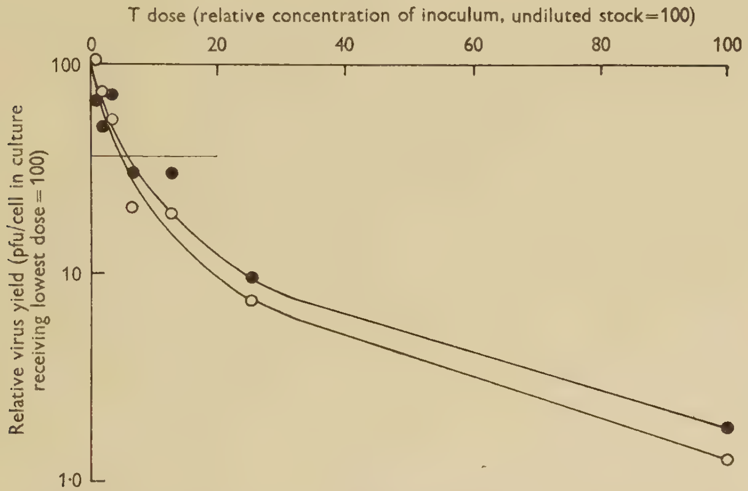

Fig. 3. Relation between yield and inoculum dilution for a T-containing stock before (○) and after (●) removal of 90% of the plaque-forming units by centrifugation (for details see Table 6). Serial dilutions of the two samples were added to monolayers in 0·5 ml. amounts, and 5 ml. medium added after 30 min. adsorption. Medium was harvested for assay after 22 hr. Relative yield is calculated on the basis of 2×10^7 cells/monolayer, although not all cells yield virus.

DISCUSSION

These results provide a little more information about the way in which the agent T excludes infective virus, and about the properties of the T particle itself. The exponential relation between dose of T and yield of virus at low doses indicates that only one T particle needs to be adsorbed to a cell for infective virus to be excluded. This high efficiency is quite different from the heterotypic interference of dilute passage vesicular stomatitis virus (Cooper, 1958*b*) where, although exclusion occurs, interfering and challenge virus appear to compete, and adsorption of many interfering particles is needed to exclude a single challenge particle; this may reflect differing rates of establishing exclusion and infection. However, at high doses of undiluted passage virus T does not protect as many cells as would be expected, perhaps because more cells receive infective virus before T has had time to establish itself. Nevertheless, even when all cells have received infective virus at an early time (Fig. 1) the relationship is still linear; thus although fewer cells have received T in time so that fewer cells are protected, the protection is still efficient in the sense that only one particle appears to be adequate.

The presence of so much T component in the medium 8 hr. after infection (Table 6) means that the production and release of T must be broadly coincident with that of infective virus.

The homotypic interfering ability of T differs from the infectivity of intact virus in its much greater stability to heat and u.v. inactivation, but resembles the heterotypic interfering ability of intact virus in this respect. It is interesting that, in heterotypic tests, T was not neutralized by antiserum. T is also much less easily sedimented in the centrifuge than is virus, indicating that it is smaller, or less dense, or both. Its consequent contamination with soluble antigen has impeded serological identification as 'virus' by this means.

The formation of T by undiluted passage (Cooper & Bellett, 1959) suggested an analogy with incomplete influenza virus, and several other properties of T support this idea. However, most of the properties found are also shared with influenza interferon (Isaacs & Lindenmann, 1957); for example, all three have lower sedimentation constants than infective virus (Gard, von Magnus, Svedmir & Birch-Andersen, 1952; Isaacs, Lindenmann & Valentine, 1957) and all are relatively insensitive to heat or u.v. inactivation (Paucker & Henle, 1958; Burke & Isaacs, 1958; Isaacs, Lindenmann & Valentine, 1957). Interferon might be expected to be found after undiluted passage of influenza virus because of the presence of more heat-inactivated virus than in dilute passage; heat-inactivated virus stimulates formation of interferon (Isaacs & Lindenmann, 1957). None of the three forms is transmissible by itself, but interferon, like T and incomplete influenza virus, should be transmissible in indefinite subculture provided that it is passed with appropriate amounts of infective virus. Neither T nor interferon (Isaacs, Lindenmann & Valentine, 1957) is sensitive to immune serum; precisely comparable data seem to be unavailable for interference by incomplete influenza virus, although one might expect it to differ in this respect. Interferon (Isaacs, 1959) and T are not visibly cytotoxic by themselves; incomplete influenza virus is also probably nontoxic (von Magnus, 1954). Although incomplete influenza virus requires an 18–24 hr. interval before challenge to establish maximum interference, appreciable interference occurred after 1 hr. (von Magnus, 1954); interferon requires several hr. incubation at 37° for its effect to be maximal (Isaacs, 1959). T probably establishes most of its interference within 1 hr. although there was evidence that interference improved for a few hours after. It is difficult to compare the rates of development of interference in such different systems, but it seems that T resembles interferon more than incomplete influenza virus in this respect.

Interference in influenza virus is undoubtedly a complex thing, and may involve even more components than those already discovered. At present there is unfortunately not enough evidence to conclude much about T by analogy with influenza virus; T could be an incomplete form, or an interferon, or some other entity quite different from these, although where comparison is possible there is more resemblance with interferon, particularly in its slow rate of sedimentation in the centrifuge.

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Changes in Cellular Ribonucleic Acid during Growth of Vesicular Stomatitis Virus in Chick Cell Culture

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SUMMARY: The nuclei and small-particle (mitochondria + microsomes) fractions, separated from *in vitro* cultured chick embryo cells during one-step growth of vesicular stomatitis virus, were fractionated into RNA nucleotides by NaCl extraction, hydrolysis and paper electrophoresis. This allowed determination of molar and specific activity ratios of the RNA nucleotides during complementary ^{32}P 'gain' and 'loss' experiments. Early exponential virus release coincided with a significant increase in uridine content of nuclear and small-particle RNA over uninfected values, and a decrease in specific activity of small-particle RNA uridylic acid in ^{32}P gain experiments. Other fractions showed no change. This suggested a relatively large synthesis of an RNA different from cellular RNA and containing a high proportion of uridine at a time when virus nucleic acid synthesis was probably at its peak. This synthesis appeared to be balanced by a simultaneous breakdown of pre-existing RNA.

The study of pathways of phosphorus metabolism in chick embryo cells infected with vesicular stomatitis (VS) virus has shown an inhibition of phosphorus incorporation into the components of the infected cells (Cooper, 1957*b*). This inhibition appears to begin with the period of exponential virus release. The total amounts and the proportion of the various cellular phosphorus fractions, however, do not change appreciably. The absence of important changes in the various phosphorus fractions may be interpreted in two ways. Either the synthesis of viral nucleic acid and protein involves a very small fraction of the total cellular nucleic acid; or the cellular nucleic acid turns over internally without any over-all changes in amount. Whichever change occurs, the synthesis of new nucleic acids may cause detectable changes in purine or pyrimidine base ratios and in nucleotide specific activities; possibly it might lead to the appearance of some unusual base. The localization of such changes might also indicate the site of virus reproduction. The results given below confirm the smallness of the over-all changes produced by infection, but show significant changes in ribonucleic acid (RNA) base composition. These results suggest an appreciable and perhaps large synthesis of a type of RNA different from that in uninfected cells, and occurring during the early exponential virus release period, while pre-existing RNA is simultaneously broken down.

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METHODS

General type of experiment. The RNA components of different particulate fractions of infected and non-infected cells were compared at different times during the latent and early release period of one-step ^{32}P 'gain' and 'loss' experiments (Cooper, 1957*b*). Since Cooper found that deoxyribonucleic acid incorporated or lost practically no ^{32}P during the infective process, the analyses were limited to the RNA. The sucrose-soluble fraction as obtained contained too low a concentration of RNA to be handled satisfactorily by present methods; only a few assays were performed on this fraction. In general these assays yielded results very similar to those obtained with the small-particle fraction. Simultaneous virus assays were performed on the medium, and gave results identical with those to be expected from the reproducible type of experiments described by Cooper (1957*a*) which were performed in the same laboratory at the same time as the experiments described here. Thus the times and conditions of infection in the two sets of work are closely comparable. The preparation of stocks and of tissue cultures, the conditions of infection, the 1-step growth-curve method, the virus assay method and the media used were described by Cooper (1957*a*).

^{32}P gain experiments. Six experiments of this type were carried out, representing six time-points during the growth cycle. Each experiment involved 30 monolayer cultures of cells from 10-day chick embryos, used when 24 hr. old and containing *c.* 2×10^7 cells. The medium was removed and 15 cultures were completely infected with $2–3 \times 10^8$ plaque-forming units (pfu) of vesicular stomatitis virus in 0·5 ml. medium (average multiplicity of infection = 10 pfu/cell), the remainder receiving 0·5 ml. medium as control. The cultures were incubated at 37° in a 5% (v/v) CO_2-air mixture for 30 min., when 1–3 μc. ^{32}P phosphate in 5 ml. medium were added to each infected and control culture. At times appropriate to each experiment during a further 2–6 hr. period at 37°, the medium was removed for virus assay and the cells of all cultures washed with Earle's saline (Earle *et al.* 1943) and quantitatively collected from the dishes with 2·5 mg. trypsin/ml. in Earle's saline; all control cultures and all infected cultures of any one experiment, respectively, were pooled to make one large control and one large infected cell sample for each time-point.

^{32}P *loss experiments.* Four experiments of this type were performed, using the same number and kind of monolayers as in the gain experiment. Without removing the medium, ^{32}P phosphate was added (1–3 μc./monolayer) to 24 hr. cultures which were replaced at 37° for 12 hr. and then washed three times with Earle's saline. Half of the cultures were infected as before, the remainder receiving medium only. After 30 min. to allow for virus adsorption 5 ml. fresh medium were added, and the cells harvested and pooled as before at various intervals between 3 and 6 hr. after infection.

Separation of cell particulate fractions. In both gain and loss experiments the harvested cells were washed twice with 0·25 M-sucrose and homogenized in less than 0·3 ml. of 0·25 M-sucrose, according to the method of Hogeboom, Schneider

& Striebich (1953). By differential centrifugation three fractions were collected: (*a*) nuclei, sedimented for 5 min. at 700 *g*; (*b*) mitochondria + microsomes (here called small-particle fraction), obtained from the supernatant fluid of the nuclei by sedimenting together for 25 min. at 60,000 *g*; (*c*) the final supernatant fluid (soluble fraction).

Separation of chemical fractions. The method of Davidson & Smellie (1952) was used for the separation and the quantitative determination of the ribonucleotides in the various fractions. After 1 hr. precipitation with trichloroacetic acid (10 %, w/v) at 4°, followed by two washings of the precipitate with trichloroacetic acid, lipids were extracted with ethanol + chloroform mixture (3:1, v/v) at 75° three times for 10 min. each time, and with ether twice at room temperature for 15 min. each time. The residue was extracted three times with 10 ml. of 100 g. NaCl/l. solution for 1 hr. at 100°. The extract (30 ml.) was precipitated overnight at 0° with 60 ml. of 10 % (v/v) acetic acid in ethanol containing 10 mg. $MgCl_2$. The precipitate was centrifuged down, redissolved in 4 ml. water and centrifuged to remove the insoluble residue. The nucleic acid was then precipitated from the clear supernatant fluid by addition of 8 ml. ethanol, washed with 2 ml. ethanol, dried with ether and hydrolysed with 0·3 N-KOH at 37° overnight. This solution was brought to pH 1 with $HClO_4$ and the precipitate of DNA, protein and $KClO_4$ discarded. The supernatant fluid was concentrated under a current of air and the pH brought to 3·5–4·0 with 0·1 N-KOH. Volumes (30–50 μl.) of this hydrolysate were separated by paper electrophoresis for 4 hr. at 700 V. (Davidson & Smellie, 1952); the spots were identified by ultraviolet (u.v.) photography (Markham & Smith, 1949, 1951) cut out and eluted with 0·1 N-HCl at room temperature for 24 hr.

The quantity of nucleotide in each eluate was determined by a Beckman u.v. spectrophotometer and the radioactivity measured with a Nuclear Instrument and Chemical Corp. (Chicago) automatic end-window Geiger counter, making allowance for background counts and radioactive decay as usual. The determinations were made at the following wavelengths: adenylic acid 257 mμ, cytidylic acid 278 mμ, guanylic acid 257 mμ, uridylic acid 262 mμ, both for the spots and for corresponding blanks cut in the paper in similar positions; the extinctions were divided by the following values to obtain the millimolar (mM) concentrations: adenylic acid 14·4, cytidylic acid 13, guanylic acid 12·4, uridylic acid 9·9. From the nucleotide concentrations the nucleotide P concentrations were calculated, giving with radioactivity assays the specific activity (as counts/min./μg. P).

RESULTS

All data are considered by comparing infected with uninfected (control) values at various stages during a virus growth cycle. The specific radioactivity data are treated in two ways, either by comparing with controls the absolute values of the specific activities of the individual nucleotides derived from the different RNA's, or by comparing nucleotide specific activity ratios (adenylic acid = 10). The nucleotide compositions are compared as molar ratios (adenine = 10). In comparing the specific activity and molar ratios of the four nucleotides, advantage was taken of the fact that the corresponding ratios in control cultures did not change with time of incubation, so that all ratios, both control and infected, are presented in terms of the average of uninfected values (= 100).

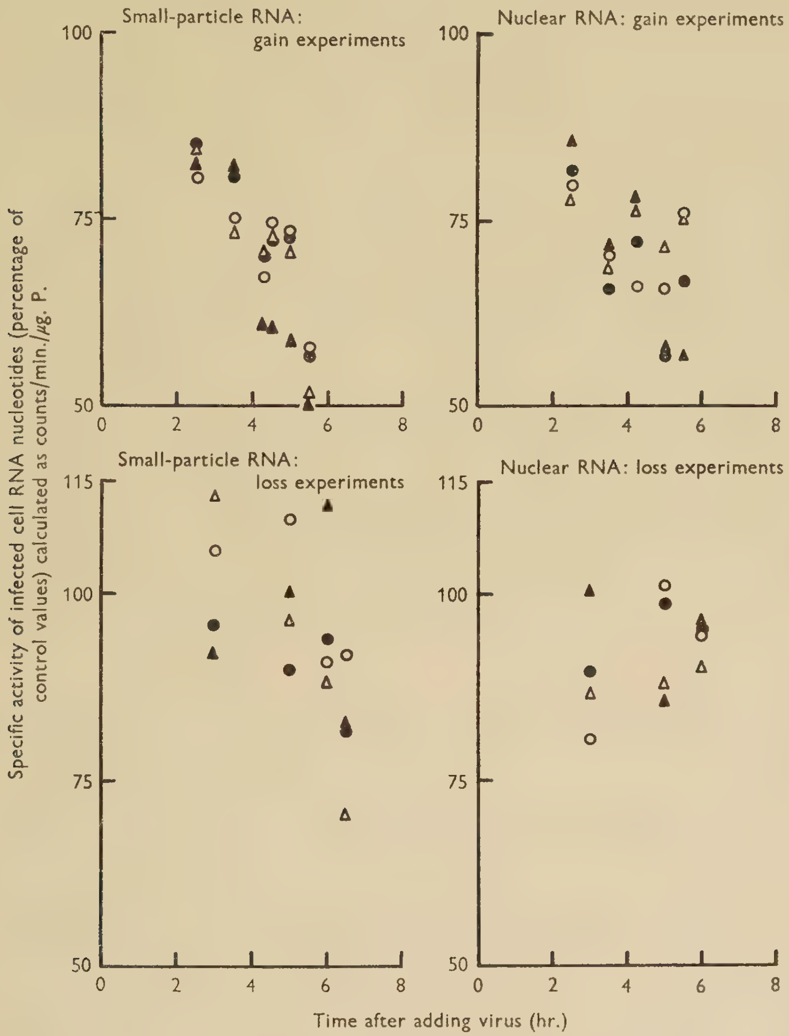

Fig. 1. The effect of vesicular stomatitis virus infection on the absolute specific activities of nucleotides extracted from RNA of small-particle and nuclear fractions during ^{32}P gain and loss experiments. The experiments are the same as those of Tables 1 and 2, where specific activity ratios (adenylic acid = 10) rather than absolute values are presented. The absolute specific activities are calculated in terms of counts ^{32}P/min./μg. P, and are expressed as percentages of the corresponding control values. ○, cytidylic acid; ●, adenylic acid; △, guanylic acid; and ▲, uridylic acid.

Table 1. *Effect of vesicular stomatitis virus infection on the molar and specific activity (SA) ratios of nucleotides derived from RNA nuclear and small-particle fractions during ^{32}P gain experiments*

The ratios are calculated in terms of adenylic acid = 10, and infected cultures (= V) are compared with identical but uninfected controls (= C) by expressing both as a percentage of the average ratio of the control cultures. σ = standard deviation of control cultures. The first values in each column are the average control molar or SA ratios of each nucleotide $\pm\sigma$, with which the other values are compared.

	Time after infection (hr.)	Cytidylic acid				Guanylic acid				Uridylic acid			
		Molar ratio		SA ratio		Molar ratio		SA ratio		Molar ratio		SA ratio	
		C	V	C	V	C	V	C	V	C	V	C	V
Nuclear RNA		12·86 ± 0·76 = 100		9·00 ± 0·59 = 100		16·44 ± 2·09 = 100		8·88 ± 0·62 = 100		8·24 ± 1·03 = 100		11·29 ± 0·56 = 100	
Expt. no.													
1	0	93	—	93	—	100	—	114	—	97	—	97	—
2	0	106	—	111	—	98	—	104	—	90	—	98	—
3	0	93	—	102	—	78	—	103	—	85	—	97	—
4	0	100	—	110	—	79	—	105	—	127	—	102	—
5	2·5	103	105	94	91	99	103	97	92	109	149	105	111
6	3·5	103	108	93	100	115	98	97	101	89	112	91	99
7	4·25	100	98	102	93	110	97	97	103	97	125	105	114
9	5·0	110	103	94	109	109	108	92	116	111	125	108	109
10	5·5	92	99	100	113	111	112	90	101	95	123	97	82
Mean $\pm\sigma$		100·0 ± 5·9	101·5	99·9 ± 6·6	101·2	99·9 ± 12·7	102·0	99·9 ± 7·0	102·6	100·0 ± 12·5	121·0	100·0 ± 5·0	103·0
Small-particle fraction RNA		13·4 ± 0·71 = 100		9·67 ± 0·46 = 100		17·5 ± 0·81 = 100		8·61 ± 0·61 = 100		8·2 ± 0·66 = 100		11·59 ± 0·58 = 100	
Expt. no.													
1	0	96	—	98	—	105	—	100	—	112	—	103	—
2	0	90	—	109	—	102	—	107	—	98	—	102	—
3	0	98	—	95	—	106	—	110	—	92	—	109	—
4	0	107	—	104	—	92	—	109	—	107	—	104	—
5	2·5	107	104	96	93	97	96	87	87	97	110	104	100
6	3·5	95	101	104	97	104	99	93	85	89	115	100	104
7	4·25	103	107	101	96	93	104	100	99	111	115	96	83
8	4·5	97	102	102	104	100	97	103	102	89	118	97	79
9	5·0	101	101	98	98	103	99	98	93	98	124	96	77
10	5·5	104	97	92	94	100	94	93	85	105	126	91	80
Mean $\pm\sigma$		99·8 ± 5·3	102·0	99·9 ± 4·8	97·0	100·2 ± 4·6	98·2	100·0 ± 7·1	92·0	99·8 ± 8·1	118	100·2 ± 5·0	87·0

Table 2. *Effect of vesicular stomatitis virus infection on the molar and specific activity (SA) ratios of nucleotides derived from RNA of nuclear and small-particle fractions during* ^{32}P *loss experiments*

The ratios are calculated in terms of adenylic acid = 10, and infected culture (= V) are compared with identical but uninfected controls (= C) by expressing both as a percentage of the average ratio of the control cultures. σ = standard deviation of control cultures. The first values in each column are the average control molar or SA ratios of each nucleotide ±σ, with which the other values are compared.

	Time after infection (hr.)	Cytidylic acid				Guanylic acid				Uridylic acid			
		Molar ratio		SA ratio		Molar ratio		SA ratio		Molar ratio		SA ratio	
		C	V	C	V	C	V	C	V	C	V	C	V
Nuclear RNA		10·70 ± 1·16 = 100		10·93 ± 0·95 = 100		17·30 ± 0·88 = 100		9·3 ± 0·19 = 100		8·63 ± 0·73 = 100		9·85 ± 0·14 = 100	
Expt. no.													
11	3·0	115	119	110	98	105	90	103	99	112	117	102	114
12	5·0	90	110	89	91	93	104	98	88	93	92	99	86
13	6·0	95	112	102	101	102	106	99	93	95	105	99	100
Mean ± σ		100·0 ± 10·8	113·6	100·3 ± 8·7	96·7	100·0 ± 5·1	100·0	100·0 ± 2·1	93·3	100·0 ± 8·5	104·6	100·0 ± 1·4	100·0
Small-particle fraction RNA		12·90 ± 0·38 = 100		10·25 ± 0·68 = 100		18·20 ± 0·89 = 100		8·82 ± 0·77 = 100		7·50 ± 0·38 = 100		10·3 ± 0·82 = 100	
Expt. no.													
11	3·0	104	98	97	107	105	107	87	102	99	107	106	102
12	5·0	100	93	91	112	104	101	97	104	101	119	100	112
13	6·0	99	97	109	105	96	106	110	101	93	119	87	104
14	6·5	96	93	102	115	94	103	105	91	107	117	107	107
Mean ± σ		99·8 ± 2·9	95·2	99·8 ± 6·6	109·8	99·8 ± 4·9	104·2	99·8 ± 8·7	99·5	100·0 ± 5·0	115·5	100·0 ± 8·0	106·2

Specific activities of RNA nucleotides during ^{32}P gain experiments

Nuclear RNA. The absolute specific activities progressively dropped well below control values for all nucleotides (Fig. 1); the relative specific activity ratios were unchanged (Table 1).

Small-particle RNA. The absolute specific activities also dropped well below controls for all nucleotides (Fig. 1). The relative specific activity of uridylic acid decreased consistently and significantly (Table 1), but the other nucleotides were unaffected.

Specific activities of RNA nucleotides during ^{32}P loss experiments

Nuclear RNA. The absolute specific activities were marginally lower than control (Fig. 1). There was no change in the relative specific activities (Table 2), except for a barely significant decrease in the specific activity of guanylic acid.

Small-particle RNA. There were barely significant decreases in the absolute specific activities of the last samples (Fig. 1), and no significant change in relative specific activity of any nucleotide (Table 2).

Changes in molar ratios of RNA bases during virus infection

Data from gain and loss experiments here served as duplicates.

Nuclear RNA. There was a significant and consistent increase in the proportion of uridylic acid in the gain experiments of Table 1 (not shown in the loss experiments of Table 2 which, however, probably have too few points to be significant). The other nucleotides showed no change.

Small-particle RNA. There was a significant and consistent increase in the proportion of uridylic acid in both gain and loss experiments (Tables 1 and 2), appearing 4–5 hr. after infection. The other nucleotides showed no significant change.

DISCUSSION

Four points of interest derived from these experiments may be considered. (1) There was a noticeably lower rate of ^{32}P incorporation into the nucleotides of infected cell RNA as compared with non-infected cells (absolute specific activity during gain experiments becoming lower in infected than in control cells). This effect occurred during the period of virus release and was also observed by Cooper (1957*b*) in nearly all other phosphate fractions. (2) While infection slowed ^{32}P uptake into RNA, it caused little selective early liberation of ^{32}P from pre-labelled RNA nucleotides (absolute specific activity being stable during ^{32}P loss experiments). The total amounts of RNA and nearly all other phosphate fractions also remained unchanged (Cooper, 1957*b*) indicating that early over-all lysis must be slight. (3) There were no very marked changes in the chemical composition of the RNA after infection; not only was no new base noticed, but the quantitative relationships between the pre-existing bases did not shift greatly. There was, however, a moderate but quite significant increase in the proportion of the uridylic acid in both small-particle and nuclear RNA of infected cells; this increase was accompanied by a decrease in relative specific activity of uridylic acid in the gain experiments (small-particle fraction RNA only). (4) The nucleotide specific activity ratios were a little, but usually significantly, different from unity. Without knowledge of the precursor nucleotide pools it is not possible to speculate on the meaning of this. The base ratios of the RNA of nuclear and small-particle fractions did not differ significantly, a finding which distinguishes these cells from those of, for example, rat liver, in which Crosbie, Smellie & Davidson (1953) and Elson & Chargaff (1952) found that the nuclear RNA was richer in uridylic and poorer in guanylic acids than the cytoplasmic RNA. The ratio guanylic + uridylic : adenylic + cytidylic averaged 1·1 in both control and infected cells, but there was a marginally significant difference in the ratio guanylic + cytidylic : adenylic + uridylic, which was 1·65 for infected cells and 1·53 for control cells. This was due mostly to the differences in uridylic acid.

The finding most relevant to the problem of vesicular stomatitis virus synthesis is the increase in proportion of uridylic acid in both nuclear and small-particle fraction RNA, with its decrease of relative specific activity in the small-particle fraction of the ^{32}P gain experiments. These changes cannot be due to increased synthesis of RNA uridylic acid directly from the ^{32}P of the medium since in gain experiments its relative and absolute specific activities were lower than in control cultures. The changes may represent a general breakdown of that cellular RNA existing before infection, leaving a small high-uridine residue undegraded, but the only RNA lysed by 5–6 hr. after infection (Cooper, 1957*b*) was a small part of the soluble fraction RNA (not nuclear or small-particle fractions), and it is very unlikely that this would be large enough at 4–5 hr. to account for the size of the change found. Also this would not account for the lower than normal relative specific activity of uridylic acid found in the small-particle fraction RNA of infected cells during the gain experiments.

An alternative explanation which would account for this, however, is a synthesis of a new high-uridine RNA from mono- or polynucleotides already fabricated, perhaps derived from pre-existing nucleotide pools, but possibly also directly converted (via nucleotide pools?) from pre-existing RNA. This new RNA is different from the bulk of pre-existing RNA, but could conceivably be found in small amounts in normal cells. If the new RNA is not composed exclusively of uridylic acid it must account for an appreciable portion of the RNA of the cell. Since the total RNA is neither much increased nor decreased during this significant synthesis (Cooper, 1957*b*) one must suppose that some pre-existing RNA is broken down while the new RNA is constructed, so that some interconversion of RNA is certainly likely.

The results therefore indicate that appreciable amounts of a new RNA are made and appear in both nucleus and cytoplasm at a time when synthesis of virus nucleic acid is expected to be at its height; synthesis of this new RNA appears to be balanced by destruction of some pre-existing RNA. The source of the new RNA is probably pre-existing nucleotides, either from precursor nucleotide pools or from RNA. The relationship of the new RNA to virus nucleic acid is unknown; it must be very much larger in amount than that nucleic acid which is eventually incorporated into infective virus.

Essential questions are whether or not vesicular stomatitis virus contains RNA and, if so, whether its composition is similar to that found in infected cells, particularly in its high uridine content. If the new RNA is actually virus nucleic acid, this presents a quite different picture of animal virus growth than that to be expected from the small amounts of infective virus liberated. Thus animal viruses may be more like plant and bacterial viruses than they have hitherto appeared in that, rather than involving a small synthesis, the cell may become taken up with virus material. This implies that a major difference between plant and bacterial viruses on the one hand, and animal viruses on the other, is that the latter have a relatively low efficiency of making infective particles.

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The Growth of *Acanthamoeba* sp. in a Chemically Defined Medium

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SUMMARY: A study of the nutritional requirements of *Acanthamoeba* sp. Neff. has led to the development of a chemically defined medium which contains 18 amino acids, vitamin B_{12} and thiamin, acetate, citric acid, salts and trace metals. The need for vitamin B_{12} and thiamin only is an indication that Acanthamoeba may be closely related to the phytoflagellates. Growth of the amoebae in the defined medium is similar to the growth in peptone, but slower. Glucose in high concentration replaced acetate in the defined medium.

Many protozoa can now be grown axenically in chemically defined media. The species whose nutritional requirements are known are all flagellates with the exception of *Tetrahymena pyriformis*, a ciliate. Although several members of the Amoebida can be grown axenically, none so far appears to have been grown in a medium of known chemical composition.

METHODS

Organism. The amoeba used for the present investigation was isolated by Neff (1957) from soil at Pacific Grove, California. Axenic cultures of this amoeba were obtained from Dr S. H. Hutner (Haskins Laboratories, New York), where this work on its nutrition was started.

Cultivation. According to Neff (1957) this amoeba can be grown in a variety of liquid media, the most satisfactory being one which contains Proteose peptone, glucose and inorganic salts. Dr M. M. Swann (personal communication) observed that it also grew well in Mycological peptone (Oxoid) and that 4 % (w/v) was about the optimum concentration. *Acanthamoeba* sp. has been maintained in this laboratory since 1955 in 4 % (w/v) Mycological peptone. Transfers are made every 2 weeks, but the interval may be safely prolonged to 4 weeks.

Glassware. All glassware was cleaned in concentrated sulphuric acid containing potassium dichromate, rinsed twelve times in hot tap water and then twice in glass-distilled water.

Chemicals. The amino acids, vitamins (except B_{12}), purines and pyrimidines were obtained from Roche Products Ltd.; the remaining chemicals (AnalaR quality) from British Drug Houses Ltd. Cyanocobalamin (vitamin B_{12}) was kindly supplied by Glaxo Laboratories Ltd. as their preparation 'Cytamen'. Stock solutions of chemicals were stored at 5° with volatile preservative added (Hutner, Provasoli & Filfus, 1953). The amino acids were prepared as a dry mixture (Hutner, Bach & Ross, 1956); the vitamins were similarly prepared with mannitol as triturate. Solutions of chemicals and media were prepared with twice glass-distilled water which had been stored with volatile preservative.

Media. Since *Acanthamoeba* sp. will grow in an aqueous solution of peptone which has been sterilized by autoclaving, it was assumed to require only water-soluble and heat-stable nutrients for growth. The composition of a basal defined medium (B.D.M.) containing only such nutrients is shown in Table 1. The amino acids and vitamins were added; the remaining chemicals were measured from stock solutions. Water was added to bring the basal medium to twice the desired concentration. Further nutrients could then be added in an equal volume of water to samples of the twice-concentrated medium, thereby adjusting the concentration of the constituents to that shown in Table 1.

Table 1. *Composition of basal defined medium*

	Concn (mg./100 ml.)
Block A: amino acids	
L-Arginine HCl	45·0
L-Histidine, base	32·5
DL-Isoleucine	25·0
L-Leucine	25·0
L-Lysine 2 HCl	20·0
DL-Methionine	9·0
DL-Phenylalanine	32·0
DL-Threonine	20·0
DL-Tryptophan	25·0
DL-Valine	17·5
DL-Alanine	35·0
DL-Aspartic acid	25·0
L-Cystine	2·5
L-Glutamic acid	50·0
Glycine	25·0
L-Proline	2·0
DL-Serine	35·0
L-Tyrosine	2·0
Block P: purines and pyrimidines	
Guanylic acid	1·0
Adenylic acid	0·5
Cytidylic acid	0·5
Uracil	0·1

	Concn. (μg./100 ml.)
Block V: vitamins	
Thiamin HCl	500·0
Nicotinamide	100·0
Ca-*d*-pantothenate	100·0
Pyridoxamine 2 HCl	100·0
Riboflavin	50·0
Folic acid	5·0
Biotin	0·5
B_{12}	0·1
Pyridoxine	100·0
Choline chloride	500·0
p-Aminobenzoic acid	10·0
Inositol	1000·0
Mannitol, as triturate	2400·0
	(mg./100 ml.)
Block S: salts	
$MgSO_4.7H_2O$	20·0
$CaCl_2$	1·0
KH_2PO_4	20·0
Na_2HPO_4	20·0
Trace metal solution*	1 ml./100 ml.

* 1 ml./100 ml. medium gives metal ion concentration: μg, Fe 100, Zn 22·6, Mn 65, Mo 20, Co 0·63, Cu 0·13, EDTA Na_2 1000. Salts used were all sulphates, Mo as ammonium molybdate. pH of medium adjusted to *c.* 6·5.

An aqueous extract of yeast autolysate (Y.A., Difco), which was used as supplement for the B.D.M., was prepared by suspending 10 g. powder in 100 ml. water and boiling for 15 min; water lost by evaporation was replaced. The cooled suspension was then filtered through a no. 1 Whatman filter paper. Evaporation of a sample of the filtrate to dryness and constant weight showed that it contained *c.* 60 mg. solute/ml.

The pH value of media was adjusted before autoclaving to 6·5 (B.D.H.

indicator paper, 5·5–7·0) by the addition of N-NaOH. In experiments to test the effect of pH on growth, the pH was determined electrometrically to within ±0·1 unit.

Media were autoclaved for 30 min. at 15 lb./sq.in. Glucose was autoclaved separately.

Growth tests. In the early part of this work, the amoebae were cultured in 10 ml. medium placed in 20 × 125 mm. screw-capped test tubes. The tubes were incubated at 23° and left undisturbed for 3 weeks, which was taken as the standard growth period. In most experiments the tubes were inoculated in duplicate and from the same culture. The inoculum size was adjusted to give an initial concentration of 1–5 organisms/mm.^{3}. Growth was considered to have occurred when the concentration of cells at end of the growth period was about 100 cells/mm.^{3}. In later experiments, when it was desired to follow the rate of growth in more detail, the organisms were grown in 50 ml. conical flasks which contained 10 ml. medium and which were plugged with cotton wool. Serial samples were taken from replicated cultures at intervals of 2–3 days over a period of 2–4 weeks.

Checks for sterility were made from time to time by inoculating samples of cultures into a variety of liquid and solid media which were incubated aerobically and anaerobically.

Counting. Growth was estimated by counting live amoebae in a Fuchs–Rosenthal haemocytometer. The tube or flask was shaken vigorously for a definite number of times to dislodge the cells and a sample was immediately transferred with a clean, sterile Pasteur pipette to the counting chamber. Unless the suspension contained more than 1000 cells per 3 mm.^{3}, all the organisms in 3 mm.^{3} were counted and the results expressed as the number of amoebae/mm.^{3} of medium. The counting error was small and could be neglected in counts of less than 1000 organisms. With higher counts the sample was diluted whenever this was possible. This method was tested by following the distribution of cells in the counting chamber and then applying a Poisson calculation to the data (Fisher, 1941). Nineteen samples taken from six cultures containing different concentrations of organisms (30–260 cells/mm.^{3}) were examined. The observed distribution was found to differ significantly ($P < 0{\cdot}05$) from the expected in five out of the nineteen samples. Four of the significant differences occurred in samples containing less than 100 organisms/mm.^{3}; it was concluded that the method was adequate for concentrations in the range 100–260 organisms/mm.^{3}. When replicated counts were made on single cultures in this range, differences larger than would be expected from the error of random sampling occurred with a frequency of about 1 in 10 counts. Closer agreement with the Poisson distribution might have been achieved by performing the counts on killed organisms (Neff, Neff & Taylor, 1958), since live organisms tend to form clumps or adhere to glass. In experiments on growth rate, the cultures were replicated so that the mean with its standard error could be calculated for each point on the curve.

RESULTS

Development of a defined medium. The basal defined medium (B.D.M., Table 1) did not by itself support growth. However, when it was enriched with an aqueous extract of yeast autolysate (Y.A., 8 %, v/v), the yield was 100–200 organisms/mm.^{3} at the end of 3 weeks. Since the B.S.M. did not contain a carbohydrate source of energy, acetate and glucose were tested as possible replacements for the Y.A. When acetate (0·25 %, w/v, as the sodium salt) was added to the medium it was possible to decrease the Y.A. from 8 to 4 % (v/v) and still obtain the same amount of growth. Glucose (0·25 %, w/v) had no such sparing action. Acetate was therefore added to the B.D.M. in all subsequent experiments.

In a further series of experiments the concentration of the medium as a whole, or of the separate blocks, was varied in combination with smaller concentrations of Y.A. It was found that by raising the concentration of the salts (block S) to four times that shown in Table 1, the organisms grew in presence of only 2 % (v/v) Y.A. Block S of the B.D.M. was subsequently used at this increased concentration. The results of other tests were negative and it seemed unlikely that the Y.A. could be decreased further, still less eliminated, by varying the concentrations of nutrients in the B.D.M. At the suggestion of Dr M. R. Droop (the Marine Station, Millport), citric acid (1000 μg.) was substituted for ethylenediamine tetra-acetic acid, di-sodium salt (EDTA) in the trace metal solution; at the same time citric acid was added to the medium. Under these conditions the amoebae grew without the addition of Y.A. Table 2 shows that slow growth was maintained through three subcultures in the modified B.D.M. containing (0·1 %, w/v) citric acid.

Table 2. *The effect of adding citric acid to the basal defined medium**

	Amoebae/mm.^{3}				
	1st transfer (weeks)	2nd transfer (weeks)		3rd transfer (weeks)	
	3	3	4	3	4
B.D.M.	34	—	—	—	—
B.D.M. + citric acid 0·01 % (w/v)	78	20	—	11	—
B.D.M. + citric acid 0·1 % (w/v)	92	56	80	36	60

* Basal defined medium as in Table 1 with block S × 4, citric acid replacing EDTA in trace metal solution + 0·25 % (w/v) sodium acetate.

Simplification of the defined medium

(*a*) *Purines and pyrimidines.* The omission of block P did not impair growth and since the amoebae have been carried through at least twelve subcultures in media without purines and pyrimidines it is concluded that they can synthesize these substances.

(*b*) *Vitamins.* Initial experiments on vitamin requirements seemed to indicate that only vitamin B_{12} was essential for growth. However, in media prepared with B_{12} as the only vitamin, the yield of organisms dropped in the third subculture and growth was absent in the fourth. The results of further experiments, which are presented in Table 3, showed that thiamin was also essential for growth. It was subsequently confirmed that if either B_{12} (0·1 μg. %, w/v) or thiamin (0·1 mg. %) was omitted from the medium, growth ceased in the second transfer.

Table 3. *Analysis of vitamin requirements*

Tubes were inoculated from a culture which had been subcultured three times in a medium containing B_{12} as the only vitamin.

	Amoebae/mm.^{3} after 3 weeks	
	Tube A	Tube B
B.D.M.* without vitamins	2	0
B.D.M. + B_{12}	2	3
B.D.M. + B_{12} + thiamin	131	118
B.D.M. + B_{12} + riboflavin	2	2
B.D.M. + B_{12} + biotin	6	2
B.D.M. + B_{12} + folic acid	1	1
B.D.M. + B_{12} + Ca-*d*-pantothenate	2	2
B.D.M. + B_{12} + nicotinamide	1	3
B.D.M. + B_{12} + pyridoxamine	2	1
B.D.M. + B_{12} + pyridoxamine + thiamin	162	160

* B.D.M. as in Table 1 with blocks P and V omitted, block S × 4, citric acid replacing EDTA in trace metal solution, + sodium acetate 0·25 %, + citric acid 0·1 % (w/v). The vitamins were added at concentrations as in Table 1.

(*c*) *Amino acids.* Ammonium nitrate (0·25 %, w/v) was substituted for block A of the modified and simplified B.D.M. No growth occurred. The ten amino acids considered essential for the growing rat (block A: arginine to valine inclusive) were next tried, but these also failed to support growth. Each of the remaining 8 amino acids was added to a medium containing the ten essential acids. Growth occurred only in tubes containing glycine, but it was less than in the control tubes in which all 18 amino acids were present. The optimum concentration of block A was found to be three times that shown in Table 1. A detailed analysis of the nitrogen requirements is now in progress.

Hydrogen-ion concentration. The effect of pH value on growth in modified and simplified B.D.M. was tested in the range 5·5–7·5. Growth was equivalent in the tubes in which the medium had been adjusted to pH 6·0, 6·5 and 7·0, but was decreased at pH 5·5 and 7·5.

Chelating agents. When citric acid (other than that in the trace metal solution) was omitted from the simplified defined medium, a precipitate formed on autoclaving. This precipitate could be avoided by decreasing the concentration of the Ca and Mg salts from 4 and 80 mg. % (w/v) to 0·4 and 8·0 mg. %, respectively; growth was not affected. Nevertheless, when these concentrations were slightly exceeded some precipitation was likely to occur on autoclaving. When the medium contained Ca and Mg salts at 4 and 80 mg. %, the minimal concentration of citric acid required to prevent precipitation was 0·05 % (w/v); growth was not affected. Similarly, the citric acid could be increased to 0·2 % without depressing growth. However, when the concentration of citric acid was raised to 0·5 % the yield of organisms was halved as compared with control tubes which contained only 0·1 % citric acid.

It was not found possible to substitute EDTA for citric acid in the present medium and obtain optimum growth. The least concentration of EDTA required to prevent precipitation on autoclaving was 15 mg. % (w/v), but at this concentration growth was decreased. When the concentration of EDTA was increased to 30 mg. %, growth was absent. At this concentration there was enough EDTA to combine with all the Ca^{++} but not all the Mg^{++} in the medium; the addition of graded amounts of $CaCl_2$ to a medium containing 30 mg. % EDTA restored growth but the yield was still diminished.

Growth curves. It was now possible to compare the growth of *Acanthamoeba* sp. in peptone with that in a chemically defined medium. The results of growing the organisms are shown in Fig. 1. Serial counts were made on five tubes. Since the counts at zero time were likely to vary from 1 to 5 organisms/mm.^{3}, the initial part of each curve is of uncertain value and is shown with a broken line. Figure 2 shows the curves obtained when the amoebae were grown in 50 ml. conical flasks. Each point on the part of the curve representing logarithmic growth is the mean value obtained from eight flasks for each medium; thereafter only five flasks were used. Organisms used to inoculate tubes or flasks were taken from cultures already established in similar media. It was apparent that growth in the defined medium was comparable with that in Mycological peptone but that the rate of growth was slower. When enough time was allowed,

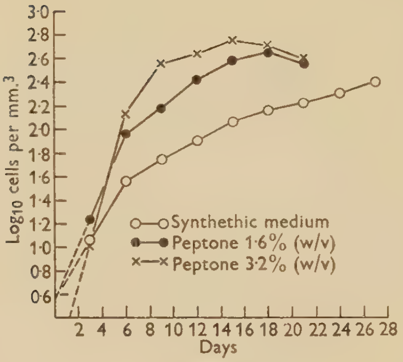

Fig. 1

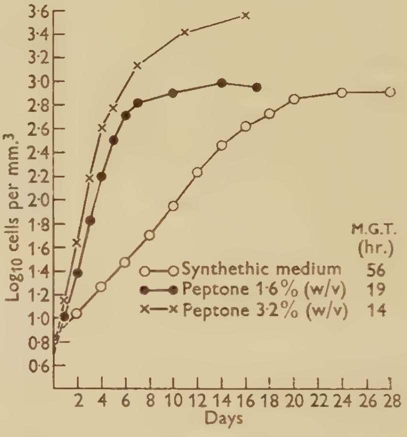

Fig. 2

Fig. 1. Growth of *Acanthamoeba* sp. in test tubes at 23° in two concentrations of mycological peptone and in defined medium. Amino acids as in Table 1, × 3, vitamin B_{12} 0·1 μg. %, thiamin 0·1 mg. %, salts as in Table 1, × 4, trace metals as in Table 1 with citric acid replacing EDTA 5 ml. %, citric acid 0·1 g. %, sodium acetate 0·1 g. %.

Fig. 2. Growth of *Acanthamoeba* sp. in flasks at 23° in two concentrations of Mycological peptone and in defined medium (composition, see Fig. 1). M.G.T. = mean generation time for logarithmic phase of growth.

the population size in the defined medium approached that in 1·6% (w/v) peptone. Growth during the log. phase could be studied satisfactorily only when the organisms were grown in flasks. The numerical results for the defined medium are shown in Table 4. Exponential growth stopped at an approximately equivalent cell concentration (300/mm.^{3}) and was independent of the nature of the medium. The total yield for each medium in flask cultures was greater than in tube cultures.

Table 4. *Growth of* Acanthamoeba *sp. in final defined medium*

Data from which log. phase of growth curve (Fig. 2) was constructed.

	Amoebae/mm.^{3}		
Days	Range (8)	Mean	S.E.
2	8– 13	11·4	0·6
4	15– 22	18·5	0·8
6	25– 35	30·0	1·0
8	42– 64	50·9	2·5
10	70–109	92·0	4·8
12	127–207	169·0	9·1
14	250–322	288·2	8·8

Table 5. *Effect of acetate and glucose on growth in tubes*

	Amoebae/mm.^{3}		
B.D.M.* plus	Range (5)	Mean	S.E.
acetate % (w/v)			
0	13– 20	18	1·3
0·05	127–181	158	8·5
0·1	158–192	176	6·4
0·25	176–228	193	9·1
0·5	97–112	104	3·2
glucose % (w/v)			
0	58– 87	77	5·2
0·1	85– 94	91	1·8
0·25	98–131	120	7·0
0·5	167–200	184	6·1
1·0	226–265	243	10·1

* B.D.M. amino acids as in Table 1 × 3, salts as in Table 1 × 4, trace metals as in Table 1 with citric acid replacing EDTA 5 ml. %, B_{12} 0·1 µg. %, thiamin 0·1 mg. %, citric acid 0·1 g. %.

Energy compounds. The information gained in the preliminary experiments suggested that glucose (0·25%, w/v) did not replace the effect on growth of Y.A. The question of the relative importance of acetate and glucose as energy compounds was therefore further studied. In two separate experiments amoebae were grown for 3 weeks in tubes containing defined medium to which sodium acetate or glucose was added in various concentrations. The results are shown in Table 5. The effect of acetate was approximately the same over the range of concentrations 0·05–0·25% (w/v). The threshold concentration was not determined but must be less than 0·05%. Growth was significantly ($P < 0.05$) diminished at the highest concentration (0·5%). The effect of glucose varied directly with the concentration. The threshold concentration under the conditions of the experiment appeared to be 0·25 % (w/v). In this same experiment were included tubes containing 0·1 % (w/v) acetate; growth (mean, 231 amoebae/mm.^{3}) did not differ significantly from that in the medium containing 1·0 % glucose. The differences in yield of amoebae in tubes without carbohydrate in the two experiments was due to the use of an aged trace-metal solution in the first experiment, where the effect of acetate concentration was tested.

An attempt was made to test the effect of acetate and glucose on the rate of growth as distinct from the effect on yield. Media were therefore distributed in flasks and counts made at 2- or 3-day intervals up to 14 days when growth was expected to be logarithmic. Counts were also made at the end of 4 weeks to obtain the population size. The results of this experiment are shown in Table 6. The rate of growth was independent of the acetate concentration in the range tested, but with glucose it appeared to vary directly with the concentration. The highest concentration of glucose tested produced the largest population but the rate of growth, as indicated by the mean generation time (M.G.T.), was less than in the media containing acetate.

Table 6. *Effect of glucose and acetate on the rate of growth in defined medium**

	Amoebae/mm.^{3}†		Estimate of
	after 2 wk.	after 4 wk.	M.G.T.‡ (hr.)
Without carbohydrate	40	189	115
Glucose 0·1 % (w/v)	67	319	110
Glucose 0·25 %	79	419	91
Glucose 0·5 %	97	654	77
Glucose 1·0 %	145	1204	70
Acetate 0·05 %	239	contam.	57
Acetate 0·1 %	292	741	55
Acetate 0·25 %	246	786	53

* Amino acids as in Table 1, ×3; salts as in Table 1, ×4; trace metals as in Table 1 with citric acid replacing EDTA 5 ml. %, B_{12} 0·1 μg. %, thiamine 0·1 mg. %, citric acid 0·1 g. %.

† Mean of counts from three flasks.

‡ M.G.T., mean generation time.

The pH value of the media at the end of 4 weeks' growth was measured electrometrically; in the flasks containing acetate it was 7·7, and in those containing glucose, 6·8. In a single experiment four flasks were set up with medium containing 0·1 % (w/v) acetate. After 2 weeks' growth the pH of two of the flasks was decreased from 7 to 6 by the addition of N-HCl. At the end of 4 weeks' growth, the counts for the treated flasks were 1184 and 1274 amoebae/mm.^{3} and for the untreated, 646 and 601 amoebae/mm.^{3}. This result showed that as the amoebae used up acetate, the accumulation of base limited the size of the population. Further, when the pH of the medium containing acetate was controlled, the yield with 0·1 % (w/v) acetate was similar to that obtained with 1·0 % glucose.

DISCUSSION

The most interesting result of this work on the nutritional requirements of *Acanthamoeba* sp. is that only two vitamins, namely B_{12} and thiamine, are essential for growth. A requirement for an exogenous source of B_{12} and thiamine is common among phyoflagellates and is regarded as characterizing auxotrophic algae (Provasoli, McLaughlin & Droop, 1957). Further, when *Ancanthamoeba* sp. is compared with the other protozoa which can be grown in chemically defined media, namely *Tetrahymena pyriformis* (Kidder & Dewey, 1951), *Herpetomonas* (*Strigomonas*) *culicidarum* (= *Crithidia fasciculata*) (Cowperthwaite, Weber, Packer & Hutner, 1953), *Leishmania tarentolae* (Trager, 1957) and *Strigomonas oncopelti* (Newton, 1957), although all need thiamine and other vitamins, Acanthamoeba is the only one which requires B_{12}. A similar comparison of purine and pyrimidine requirements shows that Acanthamoeba and *S. oncopelti* are the only species which do not need an exogenous supply of purine or pyrimidine in common with auxotrophic algae. Systematic studies have shown that all the major groups of the Phytoflagellata have some representatives which are referred to as 'amoeboid' or may have an amoeboid phase in the life cycle, whereas in the Sarcodina there are amoebae such as Naegleria, which may have a flagellate phase in the life cycle. Thus morphological observations indicate that some of the Sarcodina may be derived from the Phytoflagellata, but this is the first time that physiological evidence has been presented to substantiate this view.

Although Acanthamoeba is plant-like in respect of vitamins and nucleic acid bases, it appears to be more animal-like in respect of amino acid requirements. However, until the nitrogen requirements have been fully analysed, this point must remain uncertain.

It is common experience that one of the obstacles to obtaining growth of protozoa or algae in chemically defined media lies in finding suitable concentrations of metals (Provasoli *et al.* 1957). In the present study *Acanthamoeba* sp. grew equally well in defined media containing citric acid in the range 0·05–0·2% (w/v). At the lower concentration the ratio of chelator to added metal ion (by equivalents and excluding Na^+ and K^+) was 0·8:1. When EDTA was substituted for citric acid in the same medium at a minimal concentration (0·015%, w/v) the chelator/metal ratio was 0·12:1. The poor growth obtained in this medium did not appear to be due to under-chelation, since growth was completely inhibited when the concentration of EDTA was raised to 0·03%. However, since EDTA has a high affinity for Ca^{++} and Mg^{++} (Martell & Calvin, 1952), the EDTA available for chelation may have been largely combined with these cations and their concentration thus decreased below the optimum for growth, at the same time leaving the concentration of the heavy metals too high. Since citric acid forms less stable chelate compounds, even when it is present in excess the concentration of metal ions will be relatively high, and a metal balance favourable to growth is more readily obtained. Citric acid was shown not to be an essential metabolite but was required to keep metals in solution.

Neff *et al.* (1958) have shown that *Acanthamoeba* sp. is aeroboic with a relatively high rate of endogenous respiration (120 μl. O_2/hr./mg. N at 28°), and that it grows better in shallow than deep stationary cultures. It is thus probable that the slow rate of growth and low yield of amoebae obtained in screw-capped test tubes were due to O_2 deficiency. The same volume of medium placed in flasks supported three to six times the population, and logarithmic growth persisted for a longer period. The observation that exponential growth stopped at about the same concentration when the amoebae were grown in different media is perhaps not surprising: the amoebae are more dense than the medium and require a surface for growth in stationary cultures. An approximate estimation shows that at this concentration the amoebae, when expanded, would cover one-half of the surface available. The rate of growth could no doubt be improved if the amoebae were grown at 28°–30° which Neff *et al.* (1958) have shown to be the optimum temperature.

Neff (1957) showed that *Acanthamoeba* sp. grew only poorly in a medium of peptone and salts unless 1·0 % (w/v) glucose was added; Reich (1935) made a similar observation with *Mayorella palestinensis.* Although glucose appeared to be essential for growth, neither Neff *et al.* (1958) nor Reich (1948) was able to show stimulation of the respiration when glucose was added to suspensions of amoebae in a Warburg apparatus. A comparison of the effect of glucose and acetate on the growth of Acanthamoeba in defined medium has shown that acetate stimulates growth, and that the rate of growth appears to be independent of the acetate concentration. Glucose also stimulates growth but the rate of growth increases with the glucose concentration; and indeed, exceptionally high concentrations of glucose are required before the rate of growth approaches that obtained with acetate. The observations on the effect of acetate on growth are consistent with the view that the undissociated acetate molecule enters amoebae readily. The observations on the effect of glucose on growth suggest that this molecule does not enter the cell through the surface but only becomes available for metabolism when ingested in food vacuoles. This suggestion is supported by the experiments of Chapman-Andresen & Holter (1955) on glucose intake in *Chaos chaos*. These authors showed that uniformly ^{14}C-labelled glucose entered the cell rapidly and was metabolized only when the amoebae were induced to form pinocytosis vacuoles. The fact that many algal flagellates are able to use only a limited range of nutrients for growth may be related to the impermeability of the cell membrane. With the loss of chlorophyll and a shift towards an animal mode of nutrition, either the cell membrane must become permeable to a wider range of nutrients, or the organism could become phagotrophic. Acanthamoeba is undoubtedly phagotrophic and can be seen forming food vacuoles in fluid media. Hence the stimulant effect on growth of increases in the concentration of nutrients in the medium could be explained by ingestion of the medium rather than by the diffusion or active transport of nutrients into the cell.

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Bacterial Mutation in a Stationary Phase and the Question of Cell Turnover

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SUMMARY: During a stationary phase induced and maintained by the exhaustion of histidine, the total number of histidineless *Escherichia coli* (h −) remains constant as does the cytological appearance of the cells. If glucose is available to the starved bacteria they die at a rate of *c.* 10^{-2} per hr., while mutations to a histidine-independent (h +) condition occur at a rate of *c.* 10^{-9} per bacterium per hr. Bacteria adapted to use lactose behave essentially the same way when it, instead of glucose, is available during starvation; but if the starved cells are not fully adapted, death does not occur or is very slow (*c.* 10^{-3} per hr.) and the rate of mutation is *c.* 10^{-10}. When no carbon source is available to the starved cells, mutations cannot be detected.

The following predictions served as tests of the hypothesis of *cell*-turnover, wherein some bacteria lyse only to be replaced at the same rate by the growth of others—the mutations are presumed to have occurred during this cryptic growth:

(1) Mixtures of h^- lac^+ and h^- lac^- bacteria in lactose medium where the h^- lac^+ has a selective advantage should show population shifts if cryptic growth were occurring.

(2) During the hypothesized lysis and growth the enzyme β-galactosidase should, at predictable rates, be lost to the medium from adapted cultures in the absence of lactose and developed in unadapted cultures in its presence.

(3) Penicillin should kill those cells that grow to replace others, causing an accelerated death and preventing the mutations from taking place.

(4) The lysis and death might be microscopically observable on an agar surface.

The hypothesis of turnover did not withstand any of these tests. It was concluded, therefore, that the bacteria under investigation were not dividing and that mutations—genotypic changes (Ryan, 1955*a*)—were taking place among them.

Reasons are given to suppose that the mutations result from errors in the replication of genetic material which is in the process of turnover within the non-dividing cells.

The stationary phase has been defined as that period, following exponential growth, when there is no further increase in the mass of a bacterial culture (Monod, 1949). It is often equated with the period beginning when the number of viable bacteria determined by plating ceases to increase. In enriched media such as nutrient broth a constant level of viable cells may be retained during a period in which the mass of the culture gradually increases, the cell death rate being approximately equal to the rate of production of new cells. In chemically defined media, on the other hand, the exhaustion of sugar may cause the cessation of growth; increase in cell mass, determined as optical density, and increase in viable cell number, may cease simultaneously. During such a stationary phase there may occur a progressive decrease in viable cell number without any measurable decrease in total cell mass or number.

A similar stationary phase, in which increase in both total and viable cell number stops at the same time, is found in chemically defined media when auxotrophic bacteria are limited in their growth by the exhaustion of the growth factor they require. All other nutrients in the medium may remain in excess, at least for some time, as is shown by the fact that growth factor independent mutants will overgrow such stationary cultures of auxotrophs when they occur among the latter. This fact has been utilized to measure the rate of mutation from auxotrophy to prototrophy in *Escherichia coli* (Ryan, 1955*a*).

Mutants which require histidine (h^-) can be grown in a chemically defined medium with a limiting concentration of that amino acid. After the beginning of the stationary phase, those h^+ mutants present in some of a large number of cultures continue to grow because they do not require histidine, which has been completely consumed. If the h^- population is limited at a proper level, those cultures containing h^+ mutants can be recognized by the resultant turbidity. From the frequency of cultures without any h^+ mutants and the use of the zero term of the Poisson equation

$$P_{(0)} = e^{-m}, \tag{1}$$

the mean number of mutations/culture (m) can be calculated. With m and the average number of h^- bacteria/culture (N), the mutation rate/bacterium/generation (a) can be calculated according to the formula

$$a = \frac{m \ln 2}{N}. \tag{2}$$

A maximum estimate of the time required for single h^+ mutants to overgrow h^- cultures, during whose growth they arise, can be made by the artificial introduction of single h^+ bacteria, marked, in this case, by an inability to use lactose (lac^-). It was found that even several weeks after h^+ mutants arising during the growth of h^- populations should have overgrown (*c.* 50 hr.), new cultures adapted and became turbid. The rate of appearance of these late adaptive overgrowths was constant with time, and it could be shown that they resulted from new genotypic changes arising during the stationary phase. Phenotypic delay and the slow growth or delayed onset of division of some mutants arising during growth were eliminated as explanations (Ryan, 1955*a*). It was therefore necessary to know whether the bacteria in the stationary phase actually were in a condition in which they did not divide or whether the population was in a dynamic state of cell turnover, some bacteria lysing and yielding histidine to other viable bacteria which grew and replaced them. It could be calculated from the facts that the mutation rate (a) during growth was *c.* 3×10^{-8}/bacterium/generation, while that during the stationary phase (μ) was *c.* 1×10^{-9}/bacterium/hr., that about 2% of the h^- population would have to turn over per hr. in order for the mutations observed to have occurred during a cryptic growth (Ryan, 1955*a*).

It is the purpose of the present communication to describe evidence; derived from a study of such a stationary phase, which indicates that the bacteria do not grow and divide; there does not seem to be any turnover of cells which could account for the mutations observed.

METHODS

The stocks of *Escherichia coli* and the media employed have for the most part been described previously (Ryan & Schneider, 1949*a*). The basal medium will here be referred to as G & T; under usual circumstances it is supplemented with 25 μg. L-histidine monohydrochloride/ml. and 0·5% (w/v) glucose (H^+ medium). When lactose was used it was always filtered to sterilize through Pyrex UF fritted glass disks and added separately. Also utilized were crystalline K penicillin G (Lilly) and penicillinase (Schenley). Growth was allowed under standing conditions at 37°.

Counts of the total number of bacteria were made with a Petroff-Hausser counting chamber; viable counts were made by plating 0·1–0·3 ml. samples of appropriate dilutions on H^+ medium solidified with 2% (w/v) Difco-Bacto-agar. All figures are corrected for the volume changes that occurred during incubation of the liquid cultures despite attempts to prevent evaporation by the use of a moist incubator.

Nuclear counts were made of organisms fixed in osmic-acid vapour, hydrolysed in HCl and stained with Giemsa by usual techniques. Differential counts of lactose-utilizing (lac^+) and non-utilizing (lac^-) bacteria were made by spreading on the surface of Difco-Endo-agar.

The standard stationary phase of histidine-starved h^- bacteria was achieved by inoculating *c.* 50 washed organisms (2 centrifugations and 2 resuspensions in 0·9% (w/v) saline) into 2 ml. of G & T medium in small test tubes containing 0·5% (w/v) glucose but only 0·25 μg. histidine. Several hundred such cultures were usually employed in each experiment. Other stationary phases are described in the appropriate places.

The stock solution of artificial enrichment used contained: β-alanine, phenyl alanine, arginine, aspartic acid, glutamic acid, glycine, hydroxyproline, isoleucine, leucine, lysine, methionine, proline, serine, tryptophan tyrosine, and valine in L-form, 200 μg. of each/ml.; L-cystine, 500 μg./ml.; L-threonine, 400 μg./ml.; adenine, guanine, hypoxanthine, thymine, uracil, xanthine, adenylic acid, cytidylic acid, uridylic acid, Na guanylate, adenosine and guanosine, 200 μg. of each/ml.; choline, 50 μg./ml.; riboflavin, 4 μg./ml.; nicotinic acid, panthothenic acid P-aminobenzoic acid and pyridoxine, 2 μg. of each/ml.; folic acid, 0·2 μg./ml.; thiamin, 0·02 μg./ml.; biotin, 2×10^{-6} μg./ml. The mixture was brought to pH 7 and usually 0·1 ml. was added to 2 ml. of bacterial suspension.

The supersonic lysate was kindly prepared by Brother C. Leonard of Manhattan College. The cultures were treated for 30 min. and the h^- lac^+ bacteria employed showed a survival of *c.* 2×10^{-4}. Thus in the 0·1 ml. samples usually added to 2 ml. cultures there were *c.* 4×10^5 h^- lac^+ bacteria capable of forming colonies.

The procedure for measuring β-galactosidase activity was basically that of Cohn & Torriani (1953). Washed suspensions of bacteria were treated with 0·02 ml. toluene and 40 μg. Na deoxycholate/4 ml. and agitated at 37° for 20 min. No extraction of the enzyme was attempted. Three ml. of the resulting suspension were mixed with 0·75 ml. M/75 o-nitrophenylgalactoside in M/2 NaCl. The colour resulting from hydrolysis of this compound was measured at *c.* 27° in a Beckman spectrophotometer at 420 mμ.

RESULTS

The condition of bacteria during a growth factor controlled stationary phase was studied in experiments in which at intervals viable and total counts and cytological observations on size of organism and nuclear numbers were made. Figure 1 shows a sample of the results obtained. Although the total number of organisms remained constant, the number of bacteria capable of forming colonies on H^+ medium decreased exponentially with a rate constant (k) of *c.* 2×10^{-2}/bacterium/hr. as determined by the formulation

$$N = N_0 e^{-kt}, \tag{3}$$

where N_0 is the number of bacteria at time 0 and N the number at time t in hr. The average number of nuclei and the size of organisms remained unaltered for hundreds of hr., but the fraction of bacteria surviving eventually became so small that it was not possible to know from these observations whether that fraction differed from the remainder. No matter whether the stationary phase was induced by allowing the bacteria to remain in the medium from which they exhausted the histidine themselves or whether bacteria grown in excess histidine were washed and suspended in medium devoid of that amino acid, results of the sort shown in Fig. 1 were obtained.

Cultures containing only 10^7 bacteria/ml. barely show turbidity to the naked eye. When turbid cultures achieved by growing h^- bacteria in the presence of an excess of histidine until glucose was exhausted were investigated at different times during the stationary phase, the results shown on the top line of Fig. 1 were obtained. No significant decrease in turbidity occurred, even after 300 hr. There appeared to be no appreciable net increase in the amount of cytoplasm, and from the observations made on nuclear number it would seem that the same might have been true of DNA.

The most evident change was the loss of viability with time, which usually occurred at an exponential rate. In some experiments, after an initial phase of exponential death, the number of viable bacteria suddenly increased significantly only to decrease exponentially again. Sometimes this cycle occurred several times. It might have represented some process of recovery or perhaps a fragmentation (there is more than one nucleus per cell). It occurred even in the presence of lethal concentrations of penicillin and did not therefore result from the usual kind of growth and division of survivors. Inasmuch as it occurred only infrequently and especially because the experiments to be reported indicated that normal growth and cell division did not occur in the stationary phase, the phenomenon was not further investigated.

Under conditions of starvation for histidine such as these, where there was an excess of glucose and the salt constituents of G & T, the average rate of mutation (μ) was $1{\cdot}2\times10^{-9}$/surviving bacterium/hr. when calculated from the formulation

$$\mu=\frac{km}{N_0\,(1-e^{-kt})},\tag{4}$$

where k is the death rate constant, m the average number of mutations from time 0 to time t, and N_0 the number of bacteria at time 0 minus 27 hr., the time required from the mutational event until turbidity is manifested. This chance of mutation is constant with time (Ryan, 1955*a*).

In experiments to be reported in the next section the degree of growth was either limited by the simultaneous exhaustion of glucose and histidine or low concentrations of bacteria were suspended, after washing, in medium free from glucose and histidine. In the former case, growth of an inoculum of *c.* 50 washed h^- bacteria took place in 2 ml. lots of medium supplemented with only 0·095 μg. of histidine and 1·5 μg. of glucose per culture. The final number of bacteria per culture under these conditions was *c.* 2×10^7 and experiments in which either glucose, histidine, or both were added after growth had ceased indicated that, within the limits of measurement, both glucose and histidine were simultaneously exhausted (Table 1). Certainly after not too many hours in the stationary phase the medium was completely freed of these substances because of the metabolism of the stationary-phase bacteria. Equal-sized populations of h^- bacteria in some histidine-limited stationary phases consume in 1 hr. more glucose than was initially added to the cultures under discussion (Ryan & Schneider, 1949*b*). During this doubly limited stationary phase there was a very slow rate of death; after 300 hr. more than 60 % of the bacteria still survived. The h^+ mutants present could not grow, of course, because of the absence of glucose. After an average of 430 hr. in three experiments, when excess glucose was added, the number of adaptations observed was accountable in terms of the mutants which arose during initial growth and gave no evidence of mutations having occurred during the stationary phase. For example, when glucose was added at 41 hr., 188 of 300 cultures proved to contain h^+ mutants by adapting; when added at *c.* 430 hr., only 158 of 299 cultures adapted. When an estimate was made of the number of h^+ mutants that would survive, assuming they died during the stationary phase at the same rate as their h^- parents, it could be calculated that 150 of the original 188 cul-

Table 1. *Sample experiment demonstrating the simultaneous exhaustion of glucose and histidine from cultures initially containing 1·5 μg. of glucose and 0·095 μg. histidine and inoculated with* c. *5000 h^- bacteria*

Supplement added 66 hr. after inoculation	Time of plating (hr.)	No. of h^- bacteria developed
None	66	$2{\cdot}4\times10^7$
None	116·3	$2{\cdot}4\times10^7$
1 mg. glucose	116·3	$3{\cdot}0\times10^7$
50 μg. histidine	116·3	$2{\cdot}4\times10^7$
1 mg. glucose + 50 μg. histidine	116·3	$2{\cdot}2\times10^8$

tures containing mutants should still possess viable h+ cells. This number is not significantly different from the 158 observed after 430 hr. (P from $\chi^2 = 0{\cdot}6$). Therefore if mutations from h− to h+ did occur during this histidine- and sugar-starved stationary phase, they were not frequent. Markovich (private communication) has observed the same result.

In other experiments cultures containing *c.* 2×10^7 bacteria and devoid of glucose and histidine were made to contain 0·5 % (w/v) sterile-filtered lactose which was added after the stationary phase began. In such a stationary phase death did not usually occur even after *c.* 300 hr. (Table 2), but mutations took place at a low rate. The bacterial population usually consisted of a 50:50 mixture of h− lac+ and h− lac− strains. The h+ lac+ mutants were able to utilize the lactose present and overgrew the cultures in which they occurred, causing a visible turbidity; whatever h+ lac− mutants arose were not detected for the adaptive overgrowths were never caused by lac− bacteria. The rate of mutation/h− lac+ bacterium/hr. (μ) could be calculated by the equation

$$\mu = \frac{m}{Nt}, \tag{5}$$

where m is the average number of mutations to h+ lac+ occurring from time 0 to time t, and N is the average number of h− lac+ bacteria present during that period when death did not occur. Time 0 is set 27 hr. after the addition of lactose in order to exclude from measurement those mutants which arose during growth and which require that number of hours to overgrow (Ryan, 1955*a*). To calculate m the zero term of the Poisson formulation (1) is used, where $P_{(0)}$ is the frequency of unadapted cultures without h+ lac+ mutants.

Table 2. *The behaviour of mixtures of h− lac+ and h− lac− bacteria in a stationary phase in G & T medium where glucose and histidine were absent but* 0·5 % *(w/v) lactose was present*

The number of viable bacteria was determined by plating on Endo-agar where the lac− and lac+ colonies could be differentiated.

Time (hr.)	No. of viable bacteria	% lac+ bacteria	Total no. of bacteria
0	$2{\cdot}0 \times 10^7$	61·2	$2{\cdot}4 \times 10^7$
48·5	$1{\cdot}7 \times 10^7$	54·7	$1{\cdot}3 \times 10^7$
99·3	$2{\cdot}1 \times 10^7$	58·6	$1{\cdot}4 \times 10^7$
145·1	$2{\cdot}2 \times 10^7$	52·7	$1{\cdot}9 \times 10^7$
192·3	$2{\cdot}0 \times 10^7$	55·5	—
239·3	$1{\cdot}5 \times 10^7$	50·0	—
289·9	$1{\cdot}9 \times 10^7$	54·3	—
Av.	$1{\cdot}9 \times 10^7$	55·3	$1{\cdot}8 \times 10^7$

In five experiments where the average total number of cells per culture was $1{\cdot}6 \times 10^7$, of which 57·9 % were h− lac+, the average mutation rate (μ) was $1{\cdot}2 \times 10^{-10}$ and was constant with time (Table 3). In three experiments where the only bacteria present were h− lac+ and their number was on the average $1{\cdot}3 \times 10^7$, the average mutation rate was also $1{\cdot}2 \times 10^{-10}$. These rates are significantly lower than those found in h⁻ cultures with an excess of glucose where death occurs. The decreased rate was not caused by the failure of the h⁺ mutants to adapt to the lactose present and hence to overgrow. The addition of excess glucose or of an enrichment consisting of vitamins, amino acids and nucleic acid derivatives did not induce the overgrowth of accumulated but cryptic h⁺ mutants in histidine-starved cultures of h⁻ bacteria to

Table 3. *Constancy of the chance of mutation of h⁻ lac⁺ bacteria to the h⁺ lac⁺ condition in mixtures containing* 27·9 % *h⁻ lac⁻ bacteria and* 0·5 % (*w/v*) *lactose but no histidine*

The total number of bacteria was $1{\cdot}1 \times 10^7$/2 ml. culture.

Time in stationary phase (hr.)	Average no. of mutations (m)	Mutation rate/ bacterium/hr.
49	0·10	$2{\cdot}6 \times 10^{-10}$
72	0·15	$2{\cdot}6 \times 10^{-10}$
144	0·22	$1{\cdot}9 \times 10^{-10}$
168	0·32	$2{\cdot}4 \times 10^{-10}$

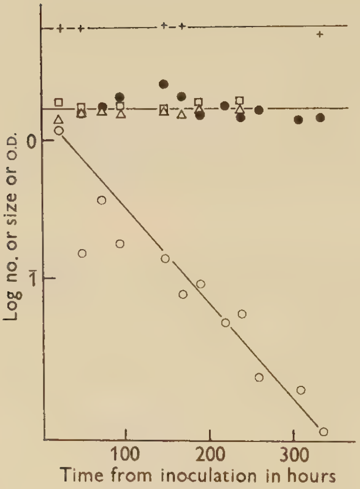


Fig. 1

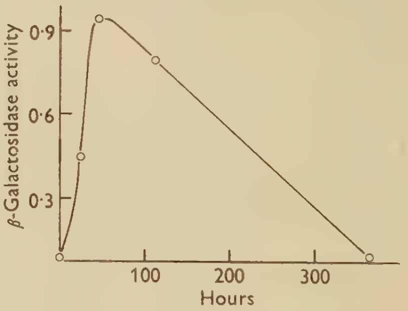


Fig. 2

Fig. 1. The properties of h⁻ bacteria in a stationary phase brought about by the exhaustion of histidine alone. About 50 h⁻ bacteria were inoculated into 2 ml. lots of G & T medium containing 0·5 % glucose (w/v) and 0·25 μg. histidine, and incubated standing at 37°. ○, number of viable h⁻ bacteria × 10^7 tested by plating on H⁺ medium; ●, total number of bacteria × 10^7 as determined in a Petroff-Hausser counting-chamber; △, average number of DNA-containing bodies/bacterium; □, average length in microns of the stained bacteria. The crosses represent the optical density in Klett units × 10^1 of 10 ml. h⁻ cultures which had been grown to a glucose-limited stationary phase in the presence of 25 μg. histidine/ml. All figures are corrected for the slight volume changes that occurred despite attempts to prevent them by the use of a moist chamber.

Fig. 2. The change in β-galactosidase activity of histidine-starved h⁻ lac⁺ bacteria in the presence of lactose. The activity is measured in optical density units/min./10^7 h⁻ lac⁺ bacteria; the abscissa is time in hr. from the addition of lactose to the glucose-grown cells.

which lactose had been added. Nor was the low mutation rate a function of a spurious survival curve due to the use of Endo-agar as a plating medium in the experiments described in Table 2, because when bacteria suspended in lactose were plated on ordinary H^+ medium with glucose the mutation rate (μ) in 16 experiments still averaged $1{\cdot}3 \times 10^{-10}$.

Under these conditions of plating there usually was a barely detectable death ($k = c.\ 2 \times 10^{-3}$). The bacteria, although initially unadapted to lactose because of their growth on glucose (residual activity, 0·02 O.D. units/min./10^7 bacteria), gradually developed β-galactosidase activity (see also Mandelstam, 1957, 1958) until a maximum of *c.* 40 % of the activity of fully adapted cells (2·6 O.D. units/min./10^7 bacteria) was achieved. Thereafter, the activity per cell decreased (Fig. 2). The h^+ mutants arising in the presence of lactose developed full activity equal to that of h^- bacteria grown in the presence of lactose. Perhaps, in contrast to glucose-grown cells suspended in glucose medium, it is the fact that a maximally vigorous metabolism is never achieved that prevents unadapted bacteria (and, of course, h^- lac^- as well) from dying in the presence of lactose and from mutating more rapidly than they do. When h^- lac^+ bacteria were grown to histidine starvation in the presence of excess lactose so that they were fully adapted to use that sugar, they underwent rapid death in the stationary phase ($k = 1{\cdot}7 \times 10^{-2}$) and their mutation rate to h^+ was $3{\cdot}8 \times 10^{-9}$/bacterium/hr. In these respects they resembled glucose-grown cells in the presence of glucose.

Table 4. *Example of an experiment in which the percentage of new growth that is* lac^+ *decreased with the time at which optimal amounts of histidine were added to histidine-starved mixtures of* h^- lac^+ *and* h^- lac^- *bacteria metabolizing lactose alone*

Time of addition of histidine (hr.)	Percentage of new growth resulting that was lac^+
0	95·4
24	90·9
44	79·6
96	72·1
140	58·1

During the stationary phase, in the absence of glucose and histidine but in the presence of lactose, a carbon source may accumulate that can be used by h^-lac^- bacteria. This was demonstrated when mixtures of h^-lac^- and h^-lac^+ bacteria in this condition were supplied with optimal amounts of histidine. The new growth that resulted may be composed of increasing percentages of lac^- bacteria as the time in the stationary phase before histidine addition increases (Table 4), but this increase could not be observed in all experiments. Although this carbon source may consist of organic acids produced from the lactose by the h^- lac^+ bacteria, its chemical nature was not identified. It was present in sufficient amounts, however, to support the maximal growth of h^- lac^- bacteria inoculated into sterile-filtered medium containing it and 0·07 μg.

of added histidine/2 ml. These bacteria might be supposed to be adapted to use this carbon source, but in the ensuing stationary phase they did not die for at least 75 hr. and they mutated to h^+ at a rate of $1{\cdot}2 \times 10^{-10}$.

Experiments to detect a possible cell turnover

How a turnover of cells occurring with a 100 % efficiency can be demonstrated directly if it takes place in the stationary phase in liquid medium is not obvious. It is possible, however, to approach the problem indirectly by several methods. The first depends upon a selective increase on the part of h^- lac^+ bacteria in a stationary phase system also containing h^- lac^- bacteria and lactose, where lysis of both types might confer a selective advantage on the h^- lac^+ component during replacement. The second involves assumptions about the enzyme β-galactosidase in adapted cells in a stationary phase devoid of lactose. A third anticipates death in the presence of penicillin of the cells that replace those assumed to have lysed. Fourth, it can be asked whether the mutations from h^- to h^+ will take place in the presence of concentrations of penicillin that will kill growing cells. Finally, direct observations of histidine-starved h^- cells on the surface of agar may be made microscopically.

Such direct observations could not be made in liquid medium containing Methocel because it was found impossible to remove contaminating traces of histidine. Similarly, the use of mechanical, absorptive, or enzymic devices to remove any histidine that might be released by the hypothesized lysis failed to illuminate the problem for reasons of technique or the interfering presence of residual histidine. Further, the steady-state concentration of histidine was, as might have been expected, too small to detect in supernatants by assay with h^- bacteria. The fraction of cells containing one or more units of the permease required for lactose assimilation would be expected to decrease upon lysis of some cells and replacement by others in the absence of an inducer (see Novick & Wiener, 1957); these experiments have not yet been completed.

Mixtures of h^- lac^+ and h^- lac^- bacteria

Cryptic growth of bacterial populations might be detected in the histidine-starved stationary phase if there were a selective advantage conferred upon the lac^+ component of a mixture of h^- lac^- and h^- lac^+ bacteria. Such an advantage might be obtained if, for example, lactose were the only carbon source present; in such an event the lac^+ component would be expected to increase in proportion as it selectively replaced the lac^- and lac^+ bacteria presumed to lyse by utilizing the histidine they released and lactose. Lactose fermentation is not extracellular, as has been shown by Benzer (1953) and as is demonstrated by the existence of sharply-sectored colonies on Endo agar.

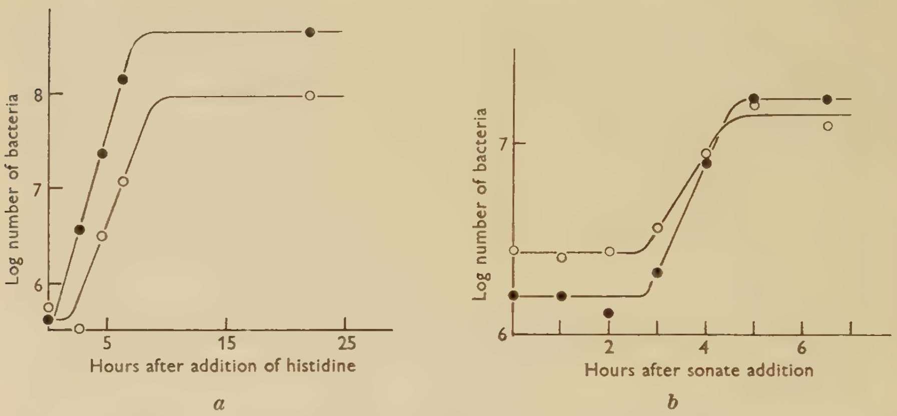
When mixtures of h^- lac^- and h^- lac^+ were grown to levels of *c.* 2×10^7/2 ml. in medium that became, as a result, exhausted of histidine and glucose and to which lactose was added, a significant shift in the proportion of the lac^+ component did not occur even after *c.* 300 hr. (Table 2). Attempts were made to determine whether there was in fact a selective advantage in favour of the lac$^+$ bacteria. Upon the addition of optimal amounts of histidine to the mixtures in a histidine-starved stationary phase, anything from 58 to 100 % of the new growth resulting, according to the time of addition, consisted of lac$^+$ bacteria (Table 4). When histidine was accompanied by an optimal amount of glucose, only 59 % of the new growth was lac$^+$; this result is understandable because both the h$^-$ lac$^+$ and the h$^-$ lac$^-$ were adapted to use glucose. In order to make the added carbon source more closely resemble that which might be produced by the hypothesized lysis but cannot be reproduced exactly, either an artificial enrichment consisting of vitamins, amino acids and nucleic acid derivatives, or an actual lysate produced by supersonic vibration, was employed. Both these enrichments supported maximal amounts of growth with no added carbon source. In the former case, $2–5 \times 10^{-1}$ μg. histidine was added either with the enrichment in the early stationary phase or some 100 hr. later. The supersonic-lysate, on the other hand, contained enough histidine to support the growth of $2{\cdot}3 \times 10^7$ h$^-$ bacteria; this histidine was limiting because, as a carbon source in the presence of excess histidine, the same volume of lysate (0·1 ml.) supported the growth of $6{\cdot}7 \times 10^7$ bacteria. Although new growth was initiated in both cases, the lac$^-$ and lac$^+$ bacteria fared equally well; they grew for the same number of generations at indistinguishable rates until the histidine was exhausted. Therefore, these experiments provided no test of the hypothesis; the β-galactosidase developed in the stationary phase conferred no advantage on the lac$^+$ bacteria.

It was otherwise when the h$^-$ lac$^+$ bacteria were previously grown on, and adapted to, lactose and then mixed in the presence of excess lactose with glucose-grown h$^-$ lac$^-$ bacteria. Either enrichment in this event allowed a selective increase in the proportion of h$^-$ lac$^+$ bacteria in the presence of limiting amounts of histidine (Fig. 3). Experiments under each condition showed that both rates of growth and numbers of generations were *c.* 1·5 × as great for the lac$^+$ component (Table 5).

When h$^-$ lac$^+$ bacteria were grown on 2×10^{-2} μg. histidine/2 ml. and 0·1 % (w/v) lactose for 70 hr. they became fully adapted to use that sugar. Upon mixing 1 ml. of them with 1 ml. of a suspension of h$^-$ lac$^-$ bacteria, grown for the same time on 2×10^{-2} μg. histidine and 1·5 μg. glucose/2 ml., in large numbers of small tubes, there remained an excess of unused lactose. After the addition of 0·1 ml. of artificial enrichment, results were obtained of which those shown in Fig. 4 are a sample. The initial proportion of h$^-$ lac$^+$ bacteria (14 %) dropped during the first 50 hr. to a level of 5 %, presumably because the h$^-$ lac$^+$, adapted to use lactose, began to die immediately through metabolizing it, while the h$^-$ lac$^-$ began to die only after some 50 hr. when they had adapted to use the enrichment. Thereafter the proportion of lac$^+$ remained constant at *c.* 3 % until after 250 hr. they seemed to have disappeared from the tubes sampled. Both h$^-$ lac$^-$ and h$^-$ lac$^+$ seemed to die with a rate constant (k) of $2{\cdot}7 \times 10^{-2}$ until sampling became inaccurate.

The fact that the excess of h$^-$ lac$^+$ predicted was not realized speaks against the hypothesis of turnover. None the less, it is possible that the rate of death of the lac$^+$ bacteria was in reality greater than that of the lac$^-$ bacteria, the apparent equality resulting from a coincidental balance through replacement by lac+ of the lysing lac− bacteria.

Even when the h− lac+ and h− lac− mixture was suspended in lactose alone without enrichment, death of the h− lac+ bacteria occurred because they were able to metabolize the lactose (Fig. 5). The rate of death (2×10^{-2}/hr.) was indistinguishable from that of h− lac+ bacteria suspended alone without h− lac− in an excess of lactose. Attempts were made, with the generous assistance of Mr Richard Levins and Dr Pinchas Mendelson, to predict the rate of decrease in the h− lac+ component of the mixed population on the assumptions that:



a *b*

Fig. 3. Increase of h− lac− and h− lac+ bacteria in histidine-starved mixtures in the presence of lactose and an enrichment upon the addition of limiting amounts of histidine. (*a*) h− lac+ bacteria grown on 2×10^{-2} μg. histidine and 0·1 % (w/v) lactose/2 ml. and h− lac− bacteria on 2×10^{-2} μg. histidine and 1·5 μg. glucose/2 ml. for 70 hr., whereupon the two strains were mixed by adding 1 ml. of each to 147 small tubes which also received 0·1 ml. of an artificial enrichment (see text); 96 hr. later 5×10^{-1} μg. histidine were added to each tube. (*b*) Bacteria grown as in (*a*) but h− lac+ on 4×10^{-2} μg. histidine; after 67 hr., 0·1 ml. supersonic lysate, which contained histidine sufficient to support the growth of $2{\cdot}3 \times 10^7$ h− bacteria, was added. ○ = lac− bacteria; ● = lac+ bacteria.

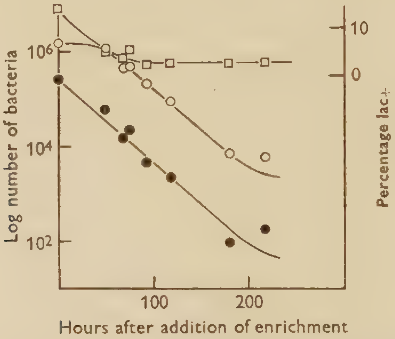


Fig. 4. The behaviour of h− lac− and adapted h− lac+ bacteria mixed in lactose and an artificial enrichment (see test). The experiment was set up as described in the caption of Fig. 3*a*. ○, h− lac− bacteria; ●, h− lac+ bacteria adapted to lactose; □, % h− lac+ bacteria.

(1) The mutations observed occurred among cells that were growing in the process of replacing other cells that had lysed.

(2) The mutation rate among these cells was the same per bacterium per generation as among cells undergoing unrestricted growth (i.e. $a = 3 \times 10^{-8}$).

(3) From these assumptions, and a knowledge of the rates of mutation of the h⁻ lac⁺ when together with h⁻ lac⁻ bacteria in a histidine-starved stationary phase and a knowledge of the number of cells per culture, the rate of replacement (β) could be calculated.

(4) The histidine released by lysis was immediately consumed by replacement so that for each component strain maintained separately the rate of replacement (β) equalled the rate of lysis (α) because the total number of bacteria remained constant.

(5) In mixed suspension the histidine released was divided among the cells present in proportion to their numbers and to the efficiencies of utilization (γ) measured in the presence of lysate and larger, but still limiting, amounts of histidine (Table 5; k_1 for h⁻ lac⁺ = 0·57; k_2 for h⁻ lac⁻ = 0·43).

Table 5. *Rates of growth and of numbers of generations passed through by h⁻ lac⁻ and lactose-adapted h⁻ lac⁺ components of histidine-starved mixtures in the presence of lactose and an enrichment upon the addition of limiting amounts of histidine*

Artificial enrichment (see text)					Supersonic lysate				
µg. histidine added to 2 ml.	Generation time (min.)		No. of generations of growth		Ml. lysate added to 2 ml.	Generation time (min.)		No. of generations of growth	
	lac⁻	lac⁺	lac⁻	lac⁺		lac⁻	lac⁺	lac⁻	lac⁺
5×10^{-1}	54	45	7·6	9·0	0·2	49	40	6·1	7·1
3×10^{-1}	68	38	6·6	10·0	0·1	55	38	2·6	3·6
2×10^{-1}	68	38	6·0	8·1	—	—	—	—	—

(6) The rates of death without lysis (k) were constant and the same as those observed when each component strain was separate.

From these we can write

$$\frac{dN^+}{dt} = k_1 N^+ - \alpha_1 N^+ + \frac{\gamma_1 N^+ (\alpha_1 N^+ + \alpha_2 N^-)}{\gamma_1 N^+ + \gamma_2 N^-}, \tag{6}$$

$$\frac{dN^-}{dt} = -k_2 N^- - \alpha_2 N^- + \frac{\gamma_2 N^- (\alpha_1 N^+ + \alpha_2 N^-)}{\gamma_2 N^- + \gamma_1 N^+,}, \tag{7}$$

where N^+ and N^- are the number of h⁻ lac⁺ and h⁻ lac⁻ bacteria.

The expressions

$$\frac{\gamma_1 N^+}{\gamma_1 N^+ + \gamma_2 N^-} \quad \text{and} \quad \frac{\gamma_2 N^-}{\gamma_2 N^- + \gamma_2 N^+}$$

are not stable enough to be considered constant even for periods as short as 100 hr. (from Fig. 5). Furthermore, since the constants for rate of lysis (α)

are not proportional to the constants for rate of utilization (γ) $(\gamma_1/\gamma_2 = 1{\cdot}5$ but $\alpha_1/\alpha_2 = 20)$ the term $(\alpha_1 N^+ + \alpha_2 N^-)/(\gamma_1 N^+ + \gamma_2 N^-)$ is not constant. The only way to preserve in the equations the linearity observed in Fig. 5 is to postulate a most complex interaction among the h$^-$ lac$^+$ and h$^-$ lac$^-$ bacteria in their utilization efficiencies and a coincidental relation between the constants.

Since there appears no biological reason to do this, we must face the fact that equations (6) and (7) predict that the rate of replacement of the h$^-$ lac$^-$ bacteria by the h$^-$ lac$^+$ would be most rapid at first and decrease with time. As a matter of fact, the slope should have almost doubled in 300 hr. and the fact that it did not indicates that the specific assumptions involved in the hypothesis of cell turnover cannot apply to the instance tested. Rather, the data indicate that the h$^-$ lac$^+$ and h$^-$ lac$^-$ bacteria in mixture neither lysed nor divided.

The gain and loss of β-galactosidase from h$^-$ lac$^+$ bacteria

In the event of cell turnover, the h$^-$ lac$^+$ cells newly formed in the presence of lactose should be induced to contain β-galactosidase. As a matter of fact, they are so induced during the histidine-starved stationary phase (Fig. 2). But whereas the β-galactosidase on the turnover hypothesis should increase at the rate of turnover (*c.* 2×10^{-3} in these experiments), in reality it increased 5 × as fast and then, unanticipated by the turnover hypothesis, decreased before the culture had become fully adapted. The adaptation of *Escherichia*

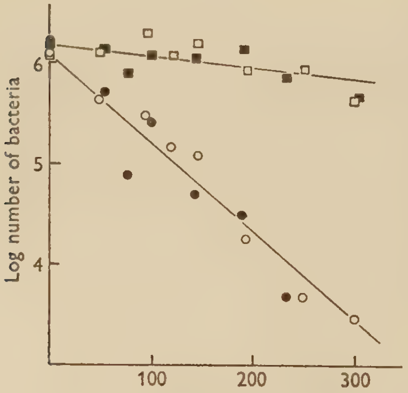


Fig. 5

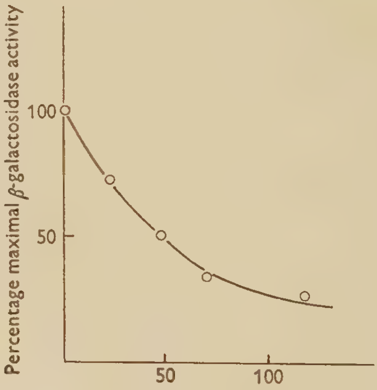


Fig. 6

Fig. 5. The behaviour of h$^-$ lac$^-$ and adapted h$^-$ lac$^+$ bacteria mixed in the presence of lactose alone. The h$^-$ lac$^+$ bacteria were grown on 2×10^{-2} μg. histidine/2 ml. and 0·1 % (w/v) lactose and the h$^-$ lac$^-$ bacteria were grown on 1×10^{-2} μg. histidine and 0·75 μg. glucose/2 ml. for 20 hr. before mixing. The squares represent the behaviour of the h$^-$ lac$^-$ bacteria and the circles of the h$^-$ lac$^+$ bacteria, in two experiments. The k for the slope of the h$^-$ lac$^+$ curve was $2{\cdot}1 \times 10^{-2}$ per hr., for the h$^-$ lac$^-$ bacteria it was $1{\cdot}2 \times 10^{-3}$. The mutation rates (μ) for the h$^-$ lac$^-$ strain averaged 2×10^{-10} and for the h$^-$ lac$^+$ strain 4×10^{-9}; the average number of bacteria per culture was 2×10^7.

Fig. 6. The loss of β-galactosidase activity from fully adapted h$^-$ lac$^+$ bacteria suspended after washing in medium where the sole carbon source was glucose. Maximal activity was 2·6 O.D./units/min./10^7 total bacteria.

coli to form *β*-galactosidase during a stationary phase has been shown by Mandelstam (1957, 1958) to occur without cell division; furthermore, the kinetics of the process is not in accord with the notion of cell turnover.

Conversely, fully adapted h⁻ lac⁺ cultures grown in lactose would, on the turnover hypothesis, be expected to lose their *β*-galactosidase at the turnover rate (2×10^{-2} in this instance) when suspended in glucose which will not induce *β*-galactosidase in cells forming in its presence. Figure 6 shows that the *β*-galactosidase activity actually does decrease in initially adapted histidine-starved h⁻ lac⁺ bacteria suspended in glucose, and at about the anticipated rate ($1{\cdot}7 \times 10^{-2}$). Such a loss has also been described by Mandelstam (1957, 1958) for non-dividing *Escherichia coli*. Furthermore, because *β*-galactosidase is an unusually stable enzyme persisting in active form in cell lysates at 37° for long times (Cohn & Torriani, 1953), that which is lost from the h⁻ lac⁺ bacteria ought to be found in their supernatants. On the contrary, supernatants from cultures of *c*. $1{\cdot}2 \times 10^7$ h⁻ lac⁺ bacteria with activities initially at a level of *c*. 2·5 and which fell in 100 hr. to less than 1·0, showed activities of 0·16 at 0 hr., 0 at 25 hr., −0·04 at 50 hr. and −0·12 at 71 hr. Clearly no stable *β*-galactosidase had been released into the medium and consequently the hypothesis of cell turnover once more was not sustained.

Addition of penicillin

The growing cells postulated by the cell turnover hypothesis should be sensitive to penicillin, in which event replacement could not take place in its presence. Therefore, in addition to the death that occurs in the absence of penicillin, there should be an additional death at least as rapid as the rate of hypothetical lysis. It would be quite abrupt, of course, if the cells killed by penicillin also released histidine that could be used for the growth of other cells.

Table 6. *The effect of penicillin on the death of histidine-starved cultures*

Treatment	Rate of death (k)	Range of k	Total no. of bacteria
None	0·028	0·015–0·038	$2{\cdot}1 \times 10^7$
Penicillin (630–1300 u.)	0·023	0·015–0·031	$2{\cdot}0 \times 10^7$
Histidine (0·125 μ)	0·038	0·018–0·071	$3{\cdot}6 \times 10^7$
Penicillin plus histidine	0·218	0·086–0·300	$2{\cdot}1 \times 10^7$
Histidine at intervals	0·028	0·017–0·039	—
Penicillin plus histidine at intervals	0·182	0·086–0·310	—

As a test of this prediction of the turnover hypothesis, *c*. 50 h⁻ bacteria were inoculated into each of several hundred 2 ml. cultures where they grew to a level of *c*. 2×10^7/culture, limited by the exhaustion of histidine. Glucose present initially at a level of 0·5 % (w/v), remained in excess. After the h⁺ mutants arising during this limited growth had overgrown the cultures (48 hr.), a lethal concentration of penicillin was added to half of the remaining tubes, the other half serving as controls. Total and viable counts were made on members of both series and the rate of death, k, was determined from the slope of exponential survival curves such as are shown in Fig. 7. The rates of death under different conditions are shown in Table 6, which embodies the averages of five experiments.

It was found that penicillin alone was without effect, the rates of death being indistinguishable in the presence and absence of that antibiotic. This suggested that none of the histidine-starved cells were in the process of division and sensitive to penicillin; if 2% of the population were turning over every hr., the k was expected to have been at least 0·06 in the presence of penicillin.

In order to be sure that the release of small amounts of histidine by lysis would cause an increased death rate, amounts of the order postulated were added with and without penicillin. A turnover of *c.* 2%/hr. requires the growth of *c.* 4×10^5 h^- bacteria/hr. (Ryan, 1955*a*) and accordingly, in the presence of excess glucose, the availability of *c.* 5×10^{-3} μg. of histidine during the interval. When this amount of histidine was added in the presence of 1300 u. penicillin/ml. no additional death was observed. Therefore, this amount of histidine, when distributed among the *c.* 2×10^7 bacteria present, did not induce enough growth metabolism to make them sensitive to penicillin. But, on the other hand, when the amount of histidine was added that would hypothetically be released in one day ($1{\cdot}25 \times 10^{-1}$ μg.) a rapid exponential death was induced in the presence of penicillin. Similarly, when lots of 5×10^{-3} μg. histidine were added every hr. for 24 hr. (total $1{\cdot}2 \times 10^{-1}$ μg.) death resulted rapidly. Since the effect of adding histidine every hour was the same as adding the sum of it all at once, it is unlikely that continual addition, which is impossible to achieve experimentally, would have given a different result. This death continued exponentially for at least 150 hr., many days after the histidine had been added.

This fact suggested that the bacteria, killed while growing on the added histidine, themselves yielded further histidine. None the less, the process is complex, for the death-rate did not accelerate as the simple conception of a chain reaction would predict and whatever penicillin 'lysis' occurred did not remove bacteria as countable units (see Trucco & Pardee, 1958) for the total cell count remained stable. During this time the penicillin remained active, for growth of the histidine-starved h^- cultures in penicillin ensued upon the addition of 28 u. penicillinase and 12 μg. histidine but not when histidine alone was added. Furthermore, in the stationary phase in the absence of penicillin (and during the previous growth) mutations occurred at characteristic rates and the h^+ mutants overgrew the cultures; in the presence of penicillin no adaptive overgrowths were observed.

It may be concluded that comparable amounts of histidine are not being released during the histidine-limited stationary phase. The presence of penicillin alone, even for more than 300 hr., did not result in increased death, although, if the rate of turnover were only 0·16%/hr., $1{\cdot}2 \times 13^{-1}$ μg. histidine should have been released. Therefore a turnover of 2 %/hr. seems out of the question and the overwhelming majority of the mutations occurring during the stationary phase could not be due to cryptic growth. A reservation would have to be put on these conclusions only if there were a threshold concentration of histidine between 1×10^{-3} and 2×10^{-3} μg/culture (1×10^{-3} μg. being contributed by the hypothetical lysis in each case) at which penicillin could induce death.

Mutation in the presence of penicillin

If mutations to h^+ took place in the presence of penicillin the resulting mutants would be unable to reveal themselves by overgrowth. As soon as the h^+ mutant began to grow its sensitivity to the penicillin present would lead to death. The h^+ mutants arising in a stationary phase can, however, be kept from growing. The method of choice is to employ h^- lac^- bacteria suspended in histidine-free medium where the only carbon source is lactose. Lac^- cells have enough residual β-galactosidase activity to allow good survival under these conditions but not enough to permit growth. The h^+ lac^- mutations occurring among such h^- lac^- bacteria can be enumerated after a long period in the stationary phase by the addition of penicillinase and glucose. Among a large number of cultures of this sort the accumulation of h^+ mutants is expressed by a decrease in the fraction of cultures without mutants.

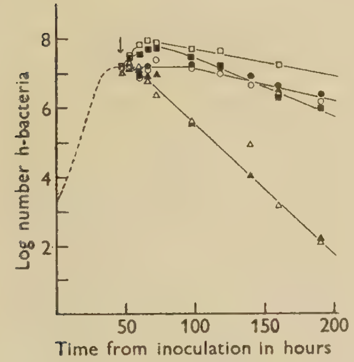


Fig. 7

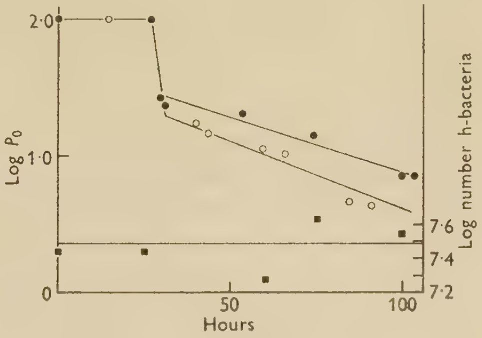


Fig. 8

Fig. 7. The effect of penicillin on the rate of death of h^- cultures starved for histidine. Penicillin and/or histidine were added at 48 hr. to cultures limited in growth by histidine exhaustion (see text for details). ○, no addition; ●, 630 u. penicillin; □, 0·125 μg. histidine; △, histidine 0·125 μg. plus penicillin; ■, 5×10^{-3} μg. histidine added every hr. for 24 hr.; ▲, 630 u. penicillin and then 5×10^{-3} μg. histidine every hr. for 24 hr.

Fig. 8. The effect of penicillin on a histidine-limited stationary phase of h^- lac^- bacteria. The ordinate on the left represents the log of the proportion (%) of non-turbid cultures; the abscissa, the time from the addition to the cultures of supplements allowing overgrowth of the h^+ mutants present. ○, cultures which had ceased growth after 48 hr. because of the simultaneous exhaustion of limiting amounts of glucose and histidine and had been supplemented with excess glucose. ●, similar cultures, supplemented at 48 hr. with lactose and penicillin instead of glucose; to which glucose and penicillinase were added 60 hr. later. ■, the number of viable h^- bacteria (right ordinate) present at different times after penicillin and lactose were added to the experimental cultures.

Several hundred 2 ml. cultures of h^- lac^- bacteria were grown to levels of $c.\ 3 \times 10^7$ cells/culture by the simultaneous exhaustion of histidine and glucose. After 48 hr., when growth was just finished on this limiting medium, sterile-filtered lactose and penicillin were added in 0·1 ml. volumes sufficiently concentrated to bring each culture to contain 0·05 % (w/v) lactose and 286 u. penicillin. Another group of cultures to which only lactose was added served as the control. Even after 278 hr. no adaptive overgrowths of h^+ mutants occurred in the control series but they were brought up on the addition of 1 μg. glucose/culture. Of course no adaptations occurred in the presence of penicillin until 40 u. penicillinase and 1 μg. glucose were added/culture. The penicillinase was free of histidine, as is shown by the fact that after its addition that h^- bacteria did not increase in number.

Among the mutants arising were those that had formed during the growth of the cultures and any that had occurred during the stationary phase. In order to determine the number of mutations that had occurred before the stationary phase, a third group of cultures received glucose at 48 hr., instead of lactose, sufficient to bring the concentration to 0·5 % (w/v). In this control group any mutants present at the time of glucose addition could overgrow rapidly. As a result, the fraction of non-turbid cultures is abruptly reduced, as shown in Fig. 8. Thereafter as mutations continued to occur in the histidine-limited stationary phase a gradual reduction in the proportion of non-turbid cultures occurred at a constant rate. The point of intersection of the two slopes gives the P_0 due to the presence of mutants arising during growth and from it the average number of mutations, m, was calculated as 1·27. This gives a mutation rate, a, during growth of 2×10^{-8}, while the rate/hr., μ, during the ensuing stationary phase was $0{\cdot}84 \times 10^{-9}$; both rates are usual for cultures with an excess of glucose.

In the series which had been in the presence of penicillin and lactose for 60 hr. and had then received penicillinase and glucose, the intersection of the two slopes was at a point equivalent to an m of 1·63. Since, as Fig. 8 shows, no death occurred in penicillin, the difference between 1·63 and 1·27 or 0·36 (approximately the displacement between the two curves) is the average number of mutations which occurred during the stationary phase in the presence of penicillin. The mutants developed in the stationary phase overgrew, along with those present when penicillin was added, as soon as glucose and penicillinase were introduced. This difference is statistically significant (P from $\chi^2 = 0{\cdot}02$) and represents a rate of mutation, μ, in the stationary phase of $1{\cdot}3 \times 10^{-10}$. In a second experiment there was some death during 115 hr. exposure to penicillin ($k_2 = 10^{-2}$) as well as in the cultures with lactose alone. The difference in m of 0·28 was enlarged therefore by correction, assuming the h^+ mutants died at the same rate as their h^- parents. The rate of mutation, μ, calculated in this way, was $5{\cdot}5 \times 10^{-10}$; the average rate of mutation in penicillin thus being $3{\cdot}5 \times 10^{-10}$/bacterium/hr. This is indistinguishable from the average rate of $1{\cdot}9 \times 10^{-10}$ found among the control cultures to which only lactose had been added.

Inasmuch as this concentration of penicillin kills growing cells (Ryan, 1955*b*) no growth through cell turnover could have occurred; yet mutations occurred at a rate normal for lactose-suspended bacteria. Therefore the mutations observed took place in the absence of growth and cell division.

Microscopic observation of starved cells

Washed cultures of h^- were spread on the surface of H^- agar and their arrangement and form were observed through the high-dry objective of a microscope and drawn for reference. The plates were incubated at 37° in a moist chamber and 2 days later the same fields were observed again. A total of 554 bacteria were studied in two experiments. Four bacteria seemed to have doubled; but the nature of bacterial division makes it impossible to be sure they were not really double upon first observation. Five extra bacteria were seen on the second observation but they were invariably near structures on the agar which might have concealed them in the first place. Seven cells were not observed at the second examination; these occurred in three groups of two and one singlet, and in each case they so resembled neighbours as to represent erroneous double registrations on the first observation. But even assuming that these 7 cells did lyse and completely disappear, the 1·3 % they represent does not nearly approach the 31 % that should have lysed to account for the mutations, which occur on agar as well as in liquid (Ryan, 1955*a*), on the turnover hypothesis. Nor were nearly enough doublings observed to replace such a large fraction of the population. It is concluded that for the most part these histidine-starved h^- bacteria neither lysed nor grew but were in a stationary phase in which they did not divide.

DISCUSSION

The hypothesis of cell-turnover in the stationary phase predicts that a fraction of the bacterial culture lyses every hour providing histidine which the surviving population may use for growth. Either the whole of the surviving population grows at a rate equal to the rate of lysis, or an equivalent fraction of the surviving population grows at a maximal rate, or something intermediate to these situations occurs. In any event, assuming the chance of mutation per generation to be the same as during unrestricted growth, the equivalent of the initial population would have been formed anew in *c.* 50 hr. ($\mu = 10^{-9}$), having grown to replace lysed cells at a faster or slower rate. As a consequence, population shifts should have been detected in the mixtures of lac^+ and lac^- bacteria; β-galactosidase should have increased and decreased in predicted ways and appeared in the culture fluid; penicillin should have killed the cultures and prevented mutation from occurring and the processes of lysis and replacement-growth should have been seen on the surface of agar. None of these anticipated events was observed. Therefore, despite the reservations that have to be placed on each test of the hypothesis, it clearly does not stand the test of experiment. Markovich (personal communication) has independently, by other methods, come to the same mind about the same system. It must be concluded that the h^- bacteria in this condition simply do not divide, or, if they do, in the face of the experiments reported, the rate of growth is not more than 10 % of that required to account for the mutations observed.

Although there may be no appreciable cryptic growth of these stationary phase populations, there well may be duplication of genetic material. There does not appear to be any significant net increase in the content of polymers such as protein, RNA and DNA but a molecular turnover of one or more of these substances may occur within the non-dividing bacteria. Thus, there is still the possibility that experiments on this system by methods such as those utilized by Meselson & Stahl (1958) will reveal that mutations arise as errors in gene replication. Indications that this may be the case can be had from the fact that the temperature relation of mutation during growth and in the stationary phase is the same (Ryan & Kiritani, 1959). The involvement of metabolism with stationary phase mutation is seen in the facts that the rate of mutation from h^- to h^+ in *Escherichia coli* growing at optimal rates is *c.* 4×10^{-8}/bacterium/hour; in the stationary phase when metabolizable sugars are the carbon source (glucose, or lactose in the case of adapted h^- lac^+) the rate is *c.* 10^{-9}, and where a poorly metabolizable sugar is the sole carbon source (lactose in the case of unadapted h^- lac^+ or h^- lac^-) the rate is *c.* 10^{-10}. In the absence of any added carbon source, or at 0° whatever the carbon source, no mutations could be demonstrated in the stationary phase (see also Markovich, 1958). Wainwright (1956) found that the extent of preincubation of the spores of Streptomyces determined the rate at which they would mutate when stored in the cold, and Stubbe (1936) observed a sudden increase in the rate of mutation in stored seeds once they reached a certain age. The non-dividing but still mutating cells of *E. coli* are considered amenable to investigating questions involving the metabolic control of mutation and in deciding whether mutations are copy-errors (Ryan *et al.* 1959). They represent not only an ageing system, but also one resembling adult somatic cells inasmuch as cell division is at a minimum.

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Stimulation of the Production of Neuraminidase in *Vibrio cholerae* Cultures by *N*-Acetylneuraminic Acid and Sialyl-Lactose

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SUMMARY: A simple medium is described which allows adequate growth of *Vibrio cholerae* with the production of only trace amounts of neuraminidase. When added to this medium to give a final concentration of 600 μM, sialyl lactose or sialyl-*N*-acetylgalactosamine, two oligosaccharides which contain ketosidically bound *N*-acetylneuraminic acid and which are substrates of neuraminidase, caused at least a 100-fold increase in the amount of neuraminidase produced during growth of the organisms. *N*-Acetylneuraminic acid was also found to stimulate neuraminidase production. Galactose and *N*-acetylgalactosamine had no effect. *N*-Acetylneuraminic acid was metabolized by *V. cholerae* as indicated by the disappearance from such enriched medium of substances giving the direct Ehrlich reaction. Evidence is presented which suggests that neuraminidase is an extracellular enzyme.

McCrea (see Burnet, McCrea & Stone, 1946; McCrea, 1947) first observed the presence in partially purified preparations of *Clostridium welchii* toxin type A of a factor able to inactivate the influenza virus receptors of human red cells, thus preventing agglutination of the cells by the virus. Culture fluids of *Vibrio cholerae* were soon found to be a richer source of the factor, which was recognized as an enzyme and referred to as the receptor-destroying enzyme (RDE; Burnet & Stone, 1947). Chemical evidence for the activity of RDE was presented by Gottschalk & Lind (1949). The glycosidase nature of the enzyme was established by Gottschalk (1956) and shortly afterwards by Heimer & Meyer (1956). When presenting evidence for the *O*-glycosidic type of the linkage susceptible to RDE, Gottschalk (1957) termed the enzyme neuraminidase and defined its action as 'the hydrolytic cleavage of the glycosidic bond joining the keto groups of neuraminic acid to D-galactose or D-galactosamine and possibly to other sugars'. The enzyme is also called sialidase (Heimer & Meyer, 1956).

Shortly after its discovery, a method for the purification of the RDE enzyme was described (Ada & French, 1950). We were unable to achieve a high degree of purification of the enzyme at that time, owing to the complex nature of the culture medium (nutrient sloppy agar) and the low enzyme titres obtained. This laboratory has since been engaged in a search for factors which, when added to a simple culture medium, would result in an improved yield of neuraminidase. Such factors have been found to be present in several biological preparations, and particularly in bovine colostrum. A preliminary examination (Ada & French, 1957) of the substance isolated from bovine colostrum indicated that it had properties similar to those of lactaminic acid-lactose (*N*-acetylneuraminyl-lactose) described by Kuhn & Brossmer (1956).

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In the present paper, a simple medium is described which allows adequate growth of *Vibrio cholerae* and provides conditions in which the neuraminidase produced is not destroyed. When added to this medium *N*-acetylneuraminic acid (*N*-ANA) and two oligosaccharides containing ketosidically bound *N*-ANA have been found to stimulate production of neuraminidase by this organism.

METHODS

Vibrio cholerae. The 4Z strain of *Vibrio cholerae* used is an old laboratory strain kindly supplied in 1946 to the Hall Institute by Professor S. D. Rubbo of the Bacteriology Department, University of Melbourne. It grows readily in nutrient broth, peptone water and on nutrient agar; large smooth colonies with clear haemolysis are produced on horse blood agar. It has been maintained as a lyophilized stock. The working stock is subcultured weekly on nutrient agar which, after overnight incubation at 37°, is stored at 4° between subcultures.

Viable counts of Vibrio cholerae. Serial tenfold dilutions were made in nutrient broth in an ice bath and four 0·05 ml. quantities of suitable dilutions allowed to dry on the surface of horse blood agar plates. Counts were made of typical colonies which developed after incubation for 48 hrs.

Titration of neuraminidase activity. An initial dilution, corresponding to the minimum value of the suspected order of magnitude of the titre, was made in calcium acetate saline, using analytical pipettes and flasks. Two sets of doubling dilutions of the initial dilution were made in the same diluent, one beginning undiluted and the other 1/3 so that the whole range of dilutions were undiluted, 1/2, 1/3, 1/4, 1/6, ..., 1/96. To one volume (0·25 ml.) of each dilution (held in tubes 75 mm. × 12 mm.) was added an equal volume of 1 % (v/v) washed fowl red cells in physiological saline, and the mixture incubated in a water bath at 37° for 60 min. The tubes were then well shaken and 0·25 ml. citrate saline containing 5 agglutinating doses of MEL influenza virus added. After mixing (by shaking) the red cells were allowed to settle at room temperature for 45 min. The dilution which showed the standard 'one plus' degree of haemagglutination was taken as the end-point. A standard enzyme preparation was included in most tests.

Influenza virus. The Melbourne strain MEL *M*, described by Isaacs & Edney (1950), was grown from limit dilution in the allantoic cavity of 11-day fertile hen eggs. Allantoic fluid virus was mixed with an equal volume of sterile glycerol. When stored at −20°, this preparation remained stable (as haemagglutinin) for at least 12 months.

Physiological saline was NaCl 0·85 g./100 ml. distilled water.

Calcium acetate saline was prepared as follows. NaCl, 5·0 g.; $CaCl_2$, 1·0 g.; CH_3COONa, 12·3 g.; N-CH_3COOH, 2·6 ml.; made up to 1 l. with distilled water.

After autoclaving (15 lb./sq.in.; 20 min.), the cooled solution had a pH value of 6·2.

Citrate saline was physiological saline containing also sodium citrate 2 g./100 ml. saline.

Collidine. A commercial preparation of γ-collidine (2:4:6-trimethylpyridine) was redistilled before use.

Determination of pH *values.* Where there was a sufficient volume ($\nless$ 2 ml.) determinations were carried out with a glass electrode. The majority of estimations were carried out on less than 0·05 ml., with indicator papers.

Ion exchange resins. Resins 1R–120 and 1R–4B were products of the Rohm Haas Co. Resin 1R–120 was regenerated with 2 N-HCl. Resin 1R–4B was regenerated with 2 N-NH_4OH. Resin Dowex 1 was a product of the Dow Chemical Co.; it was well washed with 2 N-HCl and 2 N-NaOH before use and was used in the formate form.

Estimation of sialic acid (N-*acetylneuraminic acid*; *N*-ANA). *N*-ANA was estimated by the direct Ehrlich reaction as described by Werner & Odin (1952). Crystalline *N*-ANA isolated from sialyl lactose (see below) was used as the standard reference compound.

Estimation of N-*acetylhexosamine.* The Morgan & Elson method (1934) as modified by Reissig, Strominger & Leloir (1955) was used. Crystalline *N*-acetylglucosamine, prepared from D-glucosamine HCl by the method of Breuer (1898) was used as the standard reference compound.

Paper chromatography solvents. Descending chromatography was carried out using Whatman no. 1 paper. The two solvent systems employed were *n*-butanol, pyridine and water (6+4+3) (Gottschalk, 1955); and *n*-butanol, pyridine, dimethyl formamide and water (2+1+1+1).

Paper chromatography sprays. Aniline hydrogen phthalate (Partridge, 1949); benzidine + trichloroacetate (Bacon & Edelman, 1951) and cresol green.

Isolation and identification of active factors

Sialyl lactose. Unless otherwise stated, all procedures were carried out at 0–4°. Ten litres of bovine colostrum (preferably first milking after parturition) were dialysed for 4–14 days against 3 vol. distilled water previously saturated with $CHCl_3$. The dialysate was adjusted to *c*. pH 8·5 with saturated $Ba(OH)_2$ solution, and the flocculent precipitate allowed to settle for *c*. 1 hr. The supernatant fluid, obtained by decantation and centrifugation, was stirred with 50 g. resin 1R–120 until the pH value fell to *c*. 6. The solution was passed through columns (45 cm. × 2·5 cm.) of resins 1R–120 and 1R–4B and then through a column (17 cm. × 2 cm.) of Dowex 1. The latter column was washed with 200 ml. water and then developed with 2 N-HCOOH. The effluent was collected in 4 ml. fractions, and the fractions tested with Ehrlich's reagent. The fluids which gave a positive reaction were pooled and dried *in vacuo* from the frozen state. The dried substance was dissolved in a minimum amount of methanol (insoluble material being discarded) and excess acetone added to the solution. The flocculent precipitate was washed once with acetone + methanol

(9:1) and dissolved again in methanol. After removal of insoluble material, excess diethyl ether was added to the solution and the resulting precipitate thrice washed with di:ethyl ether. Nitrogen was passed over the precipitate to remove the ether and the material finally dried to constant weight (room temperature) in a desiccator. The yield was very variable; in one experiment 3 g. was obtained.

Paper chromatography in butanol + pyridine + water gave one spot of $R_F = 0{\cdot}04$ (lactose $R_F = 0{\cdot}10$); in butanol + pyridine + dimethyl formamide + water, one spot of $R_F = 0{\cdot}37$ (lactose $R_F = 0{\cdot}42$).

Purified bacterial neuraminidase and influenza virus quantitatively split this substance into lactose and a substance reacting in the direct Ehrlich reaction (see Ada & French, 1957). The lactose moiety was identified by acid digestion (0·25 N-H_2SO_4, 16 hr., 100°) yielding equal amounts of glucose and galactose (Ada & French, 1957).

Crystalline *N*-ANA was obtained from sialyl lactose as follows: 500 mg. was dissolved in 3 ml. water and heated at 80° for 40 min. The solution was passed through a Dowex 1 column (12 cm. × 2 cm.) which was then well washed with water. The column was developed with 2 N-HCOOH, the eluate being collected in 4 ml. fractions. Those fractions which gave a positive reaction with Ehrlich's reagent was pooled and dried *in vacuo* from the frozen state, yielding a white fluffy powder. This material was crystallized and recrystallized by the method of Kuhn & Brossmer (1956). The crystals were dried to constant weight at 80° over KOH and P_2O_5.

Elementary analysis. Calc.: C = 42·7; H = 6·19; N = 4·53. Found C = 42·4, H = 6·23, N = 4·34.

Sialyl galactosamine (6-α-D-*N*-acetylneuraminyl-*N*-acetylgalactosamine) was isolated from bovine submaxillary glands by Gottschalk & Graham (1958); a sample was kindly provided by Dr Gottschalk.

Basal culture medium. In order to study the stimulating effect of the different compounds on neuraminidase production, the elaboration of a defined basal medium supporting bacterial growth without formation of neuraminidase in appreciable amounts was necessary. A further requirement was that neuraminidase produced in such a medium, containing an added active compound, should be stable. After many trials, the medium described in Table 1 was found to be satisfactory. It is a modification of that described by Feldman (1958), who in turn modified the original medium of Finkelstein & Lankford (1955). The medium had the further advantage of not interfering in the method used for the determination of *N*-ANA, *N*-acetylhexosamine and hexosamine. It also avoided the use of high concentrations of inorganic salts which had been found (French & Ada, unpublished) seriously to interfere with the yield of neuraminidase.

The medium was made up as follows. The collidine + HCl solution was made up to give a solution ten times the required final strength. This solution was sterilized by Seitz filtration. The remaining components were dissolved in water to give a solution twice the required final concentration. This solution was sterilized by autoclaving (15 lb./sq. in.; 20 min.). Appropriate volumes of the two solutions were then mixed. The complete medium (subsequently referred to as GGC medium) was stored at 2° for periods up to 3 months or for longer periods at −20°.

Experimental procedure. All samples to be tested for their ability to stimulate neuraminidase production were neutralized to pH 6·5–7·0 with NaOH and heated to 56° for 30 min. before addition to the basal medium. The medium to be inoculated was held in tubes (2–4 ml. in 1·8 × 15 cm. tubes; 5–10 ml. in 2·5 × 15 cm. tubes) which were rocked (amplitude 2·5 cm., 120 oscillations/min.) in a bath held at 37°. The medium was inoculated (at the rate of 0·05 ml./2·0 ml. medium) with a broth culture (incubated at 37°) which in turn had been inoculated from an agar slope 4–5 hr. previously. Sampling involved removal of 0·10 ml., which was then added to 0·90 ml. of a calcium acetate buffer (pH 6·2), containing 10 % (v/v) broth held in a plastic tube. The tubes were kept at −20° until the end of the experiment when one drop of toluene was added to each and the organisms lysed by four rapid cycles of freezing (*c.* −40°) and thawing (water at 20°). The tubes were centrifuged at 2000 *g* for 20 min. and the samples for enzyme estimation removed from the supernatant fluids. Two types of experiment were carried out. In the first, samples were taken 16 hr. after inoculation. In the second procedure, samples for enzyme estimation were removed at intervals during 12 hr. incubation after inoculation.

Table 1. *Composition of basal culture medium for* Vibrio cholerae *strain* 4Z

Substance	Concentration (mM)	Substance	Concentration (mM)
Glutamic acid	35	Ammonium sulphate	0·6
Glycerol	100	Magnesium sulphate	0·3
Adenine	0·3	Collidine	5
Dipotassium hydrogen phosphate	2	Hydrochloric acid	to pH 6·9

RESULTS

Decrease in pH *value of medium during growth of the organisms*

Following inoculation, the pH value of the medium decreased from 6·9 to about 6·0 after 12–16 hr. incubation at 37°. Addition to the medium of the different compounds in the concentrations reported had no effect on this decrease in pH value.

Stability of added neuraminidase in the basal medium inoculated with Vibrio cholerae

When added to a sample of the medium which had been inoculated with *Vibrio cholerae*, neuraminidase was found to be stable. Medium containing added sialyl lactose (600 μM) was inoculated with *V. cholerae* and incubated for 16 hr. at 37° as above. The culture fluid was centrifuged (2000 *g*, 20 min.) and the clear supernatant fluid removed. Part of this was heated under conditions

(80°, 30 min.) known not to affect sialyl lactose but which destroyed neuraminidase. GGC medium to which was added equal volumes of either heated or unheated supernatant fluid was prepared, inoculated and incubated as above. Samples were taken at 0 and 12 hr. In medium containing unheated fluid, the enzyme titres were: at 0 hr., 800; at 12 hr., 650. In medium containing heated fluid the titres were: at 0 hr., < 10; at 12 hr., 20.

Effect of added compounds on the growth of organisms

Addition to the basal medium of sialyl lactose, *N*-ANA or lactose to a concentration of 600 μM (the concentration used in many of the experiments to be reported) was found to have little or no effect on the growth of the organisms. Control medium and media containing added compounds were inoculated with *Vibrio cholerae* to give a final concentration of $8{\cdot}5 \times 10^5$ viable organisms/ml. After 12 hr. incubation, the viable count/ml. in the control medium was $6{\cdot}5 \times 10^8$ organisms; in the medium containing *N*-ANA, $5{\cdot}0 \times 10^8$; in the medium containing sialyl lactose, $8{\cdot}0 \times 10^8$; and in the medium containing lactose, $10{\cdot}5 \times 10^8$.

Comparison of the effects of sialyl lactose and N-*ANA on neuraminidase production*

Effect of concentration of added compounds. Sialyl lactose and *N*-ANA were found to increase the amounts of enzyme produced when added to the basal medium over the range 80–2400 μM. In both cases, the amount of enzyme produced depended upon the concentration of added compound. Details of an experiment in which enzyme titres were estimated 16 hr. after inoculation are given in Table 2.

Table 2. *Effect of concentration of* N-*acetylneuraminic acid* (N-*ANA*) *and sialyl lactose on the amount of neuraminidase produced by* Vibrio cholerae

Concentration of N-ANA or sialyl lactose (μM)	Enzyme titre in media containing	
	N-ANA	Sialyl lactose
2400	1000	2800
800	900	1800
240	400	1600
80	110	1600
Nil	50	60

Effect of subculturing Vibrio cholerae *in the GGC medium on the amount of neuraminidase produced.* When the organisms used for inoculation were subcultured several times in GGC medium instead of broth, the amount of neuraminidase produced after 12 hr. growth in the basal medium was very low. In such an experiment, GGC medium or GGC medium containing added sialyl lactose (to 600 μM) were inoculated with organisms subcultured in broth (final concentration $1{\cdot}8 \times 10^7$ viable organisms/ml.) or with organisms subcultured in GGC medium (final concentration $1{\cdot}1 \times 10^7$ viable organisms/ml.).

At 11 hr. the counts of organisms were 15×10^8/ml. and $5{\cdot}0 \times 10^8$/ml., respectively. In the tube inoculated with organisms subcultured in broth, the titres at 12 hr. were: basal medium, 70; medium + sialyl lactose, 2300. In the tubes inoculated with organisms subcultured in GGC medium, the titres at 12 hr. were: basal medium, 10; medium × sialyl lactose, 1800.

Rate of production of neuraminidase in the presence of added compounds. *N*-ANA, sialyl lactose or sialyl *N*-AGal were added to separate lots of media to give a final concentration of 600 μM. Together with a control, the media were inoculated and incubated. The enzyme titres are plotted in Fig. 1.

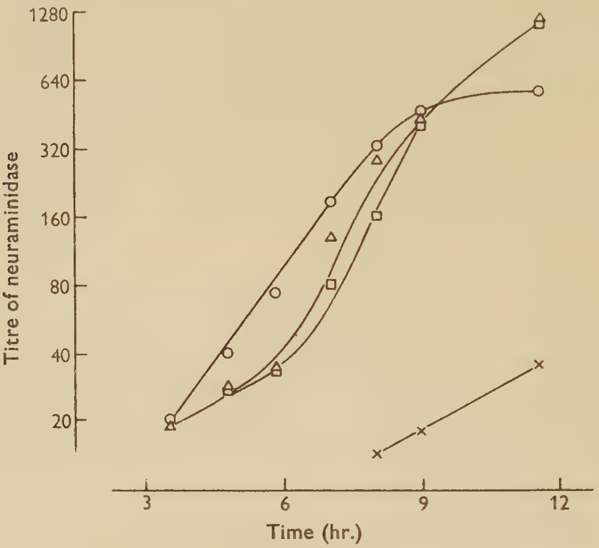

Fig. 1. Rate of production of neuraminidase in the presence of *N*-acetylneuraminic acid, sialyl lactose and sialyl-*N*-acetylgalactosamine (600 μM). Basal medium alone, ×—× ; basal medium + *N*-acetylneuraminic acid, ○—○; basal medium + sialyl lactose, △—△; basal medium + sialyl-*N*-acetylgalactosamine, □—□.

Disappearance of added active factors in media during growth of organism. Media containing either sialyl lactose (to 600 μM), sialic acid (to 600 μM) or no added factor, were inoculated and incubated for 12 hr. The direct Ehrlich reaction was used to follow the rate of disappearance of these compounds during growth of the organism. The compounds were found to be stable in uninoculated medium which had been kept for 12 hr. at 37°. The results of this experiment are presented in Fig. 2. In the medium to which sialic acid had been added, the amount of sialic acid began to decrease after the 4th hr. of incubation and had disappeared by the 8–10th hr. Samples removed at later times also gave negative reactions. Sialyl lactose was metabolized more slowly (as indicated by the reaction with Ehrlich's reagent which estimates the sialic acid portion) and some was still present at 12 hr. In a separate experiment it was found that all the sialic acid portion of added sialyl lactose had been metabolized by the 24th hr. after inoculation. In this experiment the titre of neuraminidase rose from 1600 at 10 hr. to 2300 at 24 hr.

Appearance during the breakdown of N-*ANA of a compound reacting in the Morgan & Elson reaction.* Two lots of GGC medium, one containing *N*-ANA (to 600 μM), were inoculated and then incubated at 37°. Samples were taken at intervals and the amount of *N*-ANA (direct Ehrlich reaction) and of compounds reacting in the Morgan & Elson reaction were determined. The results of one experiment are shown in Fig. 3.

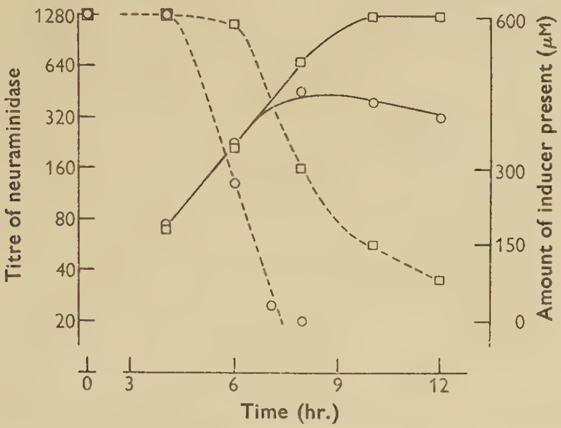

Fig. 2. Rate of production of neuraminidase and the rate of disappearances of added factors (as estimated by the direct Ehrlich test) in media enriched with *N*-acetylneuraminic acid or sialyl lactose. Enzyme production in the presence of *N*-acetylneuraminic acid, ○—○; amount of *N*-acetylneuraminic acid in medium, ○- - - -○; enzyme production in the presence of sialyl lactose, □—□; amount of sialyl lactose in medium, □- - - -□.

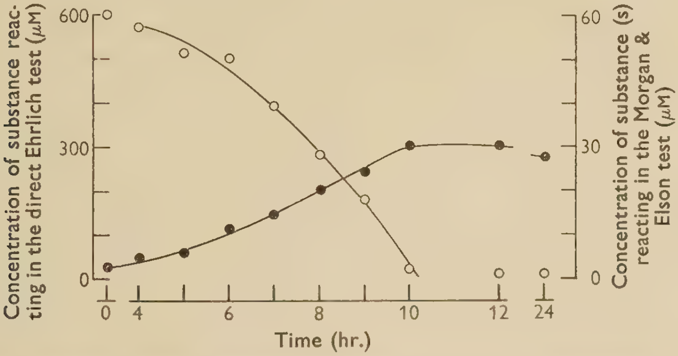

Fig. 3. Disappearance of added *N*-acetylneuraminic acid (*N*-ANA; as estimated by reaction in the direct Ehrlich test) from medium inoculated with *Vibrio cholerae* and concomitant appearance in such a culture of material which reacts in the Morgan & Elson test. Added *N*-ANA, ○—○; material reacting in the Morgan & Elson test, ●—●.

A substance which reacted in the Morgan & Elson test appeared during the breakdown of *N*-ANA. It was detected in greatest amount at the time when reaction in the direct Ehrlich test had become almost negative (*c.* 10 hr. after inoculation). The maximum amount of this factor was equivalent to a concentration of 30 μM *N*-acetylglucosamine; this result was obtained in four experiments. The stability of the substance varied, however, in different experiments. In two experiments the extent of reaction in the Morgan & Elson test remained almost unchanged between the 10th and 24th hr. In the other two experiments the extent of reaction decreased after 24 hr. incubation to 50 % or less of the maximum value reached at 10 hr. incubation.

Liberation of neuraminidase from Vibrio cholerae

At the suggestion of Dr M. R. Pollock, an experiment was carried out to see whether neuraminidase was liberated from the intact bacteria or appeared in solution only on lysis. Medium containing added sialyl lactose (600 μM) was inoculated in the usual way and incubated at 37°. At 4, 6 and 7 hr., when the medium would be expected to contain mainly viable organisms, 1 ml. samples were taken, spun at 10,000 g for 15 min. and the supernatant fluid removed. The residues were washed with 1 ml. medium and finally resuspended in 1 ml. medium. The latter was frozen and thawed four times in the presence of toluene, a procedure which was found to disrupt the bacterial cells (as shown in electron micrographs) but not to affect the titre of neuraminidase. The following are the titres of neuraminidase present in the supernatant fluids and washed cells' extracts. At 4 hr., supernatant fluid $= 40$, organisms < 1; at 6 hr., supernatant fluid $= 220$, organisms < 1; at 7 hr., supernatant fluid $= 440$, organisms $= 6$. These results suggest that neuraminidase is an extracellular enzyme.

DISCUSSION

The evidence presented in this paper indicates clearly that neuraminidase is an inducible rather than a constitutive enzyme in *Vibrio cholerae*. Organisms which were freed from traces of broth by serial subculture in the GGC medium produced only trace amounts of neuraminidase; thus an enzyme titre of 10 was found after 11 hr. incubation. In medium to which sialyl lactose had been added (600 μM), the titre at 11 hr. was 1800. Addition of inducers to the medium in this concentration did not affect the growth rate of the organism.

In studies of enzyme induction with other bacterial strains it has been established that substrates or one of the two compounds joined by the enzyme-susceptible bond may be active as inducers for the enzyme itself. A well-known example is the activity of lactose and galactose as inducers for β-galactosidase in *Escherichia coli*. Both the substrates of neuraminidase tested in the present investigations, sialyl lactose and sialyl-N-AGal, were inducers, and N-ANA, which is present in both substrates, was active. Galactose, lactose and galactosamine were inactive. The amount of enzyme produced by the organisms depended upon the concentration of inducer in the medium; this concentration dependence (over the range 2400 to 80 μM) was quite marked in the case of N-ANA but was only slight when sialyl lactose was the inducer. This aspect is being further investigated.

Heimer & Meyer (1956) showed that crude extracts of *Vibrio cholerae* contained an aldolase which split N-ANA into an N-acetylhexosamine and pyruvate. We have confirmed that *V. cholerae* can metabolize N-ANA. This was demonstrated by the disappearance of substances giving the direct Ehrlich reaction from medium containing *N*-ANA and by the appearance of material which reacted in the Morgan & Elson reaction and which reached its maximum concentration at a time when reaction with Ehrlich's reagent had almost ceased. The stability of the newly-formed substance during prolonged incubation varied. The maximum amount detected corresponded to about 5% of the maximum amount of *N*-acetylhexosamine expected (using *N*-acetylglucosamine as standard) if de-aldolization were the only pathway of degradation of *N*-ANA in the organism. Further interpretation of the data must await the isolation and identification of this substance.

We would like to thank our colleague Dr A. Gottschalk for several discussions. The work was aided by a grant from the National Health and Medical Research Council, Canberra, A.C.T., Australia.

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Stimulation of the Production of Neuraminidase in *Vibrio cholerae* Cultures by *N*-Acetylmannosamine

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SUMMARY: Addition of *N*-acetylmannosamine to a basal culture medium inoculated with *Vibrio cholerae* strain 4Z stimulated the production of neuraminidase. *N*-Acetylglucosamine and *N*-acetylgalactosamine under similar circumstances were inactive. *N*-Acetylmannosamine, added to medium inoculated with *V. cholerae*, persisted for at least 12 hr; under similar conditions *N*-acetylglucosamine was rapidly metabolized. Both D-mannosamine HCl and D-glucosamine HCl resisted enzymic attack by *V. cholerae*. The former compound severely inhibited growth of the organisms; the latter had no such effect. These findings are discussed, particularly in their relationship to the discovery that *N*-acetylneuraminic acid, which is a condensation product between *N*-acetylmannosamine and pyruvate, also induces neuraminidase.

In the preceding paper (French & Ada, 1959) it was shown that addition of sialyl-*N*-acetyl-D-galactosamine, sialyl lactose or *N*-acetylneuraminic acid (*N*-ANA) to a simple medium stimulated the production of neuraminidase by *Vibrio cholerae* without affecting the rate of growth of the organism. With the two latter compounds at least, the sialic acid portion was metabolized by the bacteria. Free *N*-ANA was broken down rapidly, and during this period the production of a small amount of a compound, possibly an *N*-acetyl hexosamine, was detected in the medium. Gottschalk's proposed structure of *N*-ANA as an aldol condensation product of an *N*-acetylated hexosamine with pyruvic acid (Gottschalk, 1956) is now generally accepted. Based on this interpretation, Cornforth, Firth & Gottschalk (1958) were successful in synthesizing from *N*-acetylglucosamine and oxaloacetic acid a compound which, on physical and chemical grounds, was identical with *N*-ANA isolated from bovine submaxillary gland mucin. Comb & Roseman (1958) have since shown that an aldolase from *Clostridium perfringens* breaks down *N*-ANA, isolated from human plasma, to pyruvate and an *N*-acetylated hexosamine identified as *N*-acetylmannosamine. Furthermore, incubation of *N*-acetylmannosamine and pyruvate with this enzyme yielded a substance which gave the direct Ehrlich reaction and which behaved like *N*-ANA when chromatographed on paper. No such substance appeared to be formed when pyruvate and either *N*-acetylgalactosamine or *N*-acetylglucosamine were incubated with the enzyme.

Earlier work (Ada & French, 1957) had indicated that addition of equimolecular amounts of *N*-acetylglucosamine and pyruvate to a peptone medium did not result in an increase in the concentration of neuraminidase over that produced in the control peptone medium. As quite appreciable amounts of neuraminidase were produced by *Vibrio cholerae* grown on the control peptone medium, this might possibly have masked the increased production of small amounts of enzyme by the added compounds. It seemed therefore appropriate to repeat this work with a basal medium which gave rise to only low titres of neuraminidase, but supported adequate growth of the organism. Furthermore, the results of Comb & Roseman (1958) made it desirable to investigate also the effect of added *N*-acetylmannosamine and pyruvate. The present paper reports a study of the activities of the *N*-acetyl derivatives of D-glucosamine, D-mannosamine and D-galactosamine and of D-mannosamine HCl and D-glucosamine HCl. *N*-Acetylmannosamine was found to stimulate neuraminidase production—the other compounds being inactive. The mode of action of *N*-acetylmannosamine in this respect, however, remains obscure.

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METHODS

Details of the basal medium used, the methods of growing *Vibrio cholerae*, counting viable organisms, estimating neuraminidase and *N*-ANA were described in the previous paper (French & Ada, 1959).

Chromatography. The solvents used were described previously (French & Ada, 1959). *N*-Acetylhexosamines were detected on paper using the spray described by Partridge (1948).

N-Acetylhexosamines. Crystalline *N*-acetylglucosamine was prepared from D-glucosamine HCl by the method of Breuer (1898). The product moved as a single component ($R_F = 0{\cdot}45$) when chromatographed with butanol + pyridine + water (6:4:3) as solvent. Crystalline *N*-acetylgalactosamine was a gift from Dr A. Gottschalk. Crystalline and amorphous *N*-acetylmannosamine preparations were generously supplied by Dr S. Roseman. The crystalline material ran as a single component ($R_F = 0{\cdot}50$) when chromatographed in butanol + pyridine + water (6:4:3). *N*-Acetylhexosamines were estimated by the Morgan & Elson method (1934) as modified by Reissig, Strominger & Leloir (1955).

Hexosamine. D-Glucosamine-HCl (British Drug Houses Ltd.) was used. Crystalline D-mannosamine-HCl was kindly supplied by Dr Roseman. Total hexosamine was determined after acid hydrolysis of the sample (2 N-HCl, 100°, 6 hr.).

Influenza viruses. The following virus strains were used: Mel—Melbourne strain of influenza A isolated in 1935; WSE—classical strain WS of influenza A virus adapted to growth on the chorioallantoic membrane of chick embryos; PR8—classical strain of influenza virus; CAM—influenza A prime strain isolated in Melbourne in 1946; Swine—Shope's strain 15 of swine influenza virus; Lee—classical influenza B strain; Mil B—influenza B strain isolated in Melbourne in 1945.

The viruses were inoculated into the allantoic cavity of 11-day fertile hen eggs and after incubation for 2 days at 35° the allantoic fluid virus was harvested and either mixed with equal parts of glycerol and stored at −20° before use or used within 24 hr. without glycerol and storage.

Calcium acetate saline and citrated saline; see French & Ada (1959).

Determination of neuraminidase receptor gradient. This was determined by incubating for 45 min. 0·1 ml. of washed packed red cells with dilutions of the enzyme under test in 4 ml. calcium acetate saline. After incubation, 0·4 ml. of 20 % sodium citrate solution was added to each tube. The red cells were centrifuged down, washed once with citrated saline and finally resuspended in 10 ml. citrated saline to give a 1 % (v/v) red cell suspension. To one volume of each suspension of treated cells, five agglutinating doses of the different viruses in one volume of normal saline were added and the red cells allowed to settle at room temperature.

Experimental procedure. The experiments to be reported were carried out as described previously (French & Ada, 1959). Media were inoculated with organisms subcultured in broth and incubated at 37°. In one type of experiment samples for enzyme estimation, etc., were taken after 16 hr. incubation; in the other experiments samples were taken from media at various times up to 12 hr. after inoculation.

RESULTS

Effect of added pyruvate and N-*acetylhexosamine on neuraminidase production*

Equimolecular amounts of sodium pyruvate and an *N*-acetylhexosamine, (either *N*-acetylglucosamine, *N*-acetylgalactosamine or *N*-acetylmannosamine), were added to portions of the medium to give final concentrations ranging from 2400 to 80 μM. The effect on neuraminidase production is shown in Table 1.

Table 1. *Effect of added* N-*acetylhexosamine and pyruvate on the amount of neuraminidase produced by* Vibrio cholerae

Enzyme titres in media inoculated with *Vibrio cholerae* were determined after 16 hr. incubation at 37°.

Concentration of *N*-Acetylhexosamine and pyruvate (μM)	Media containing *N*-acetylglucosamine + pyruvate	*N*-Acetylgalactosamine + pyruvate	*N*-acetyl mannosamine + pyruvate
	Enzyme titre		
2400	10	15	320
800	20	25	200
240	15	10	130
80	15	15	80
Nil	40	10	< 10

Stimulation of neuraminidase production by N-*acetylmannosamine without added pyruvate*

The presence of *N*-acetylmannosamine alone (no added pyruvate) in the basal medium was found to stimulate neuraminidase production. Details of such an experiment are given in Table 2. In an experiment similar to that described previously (French & Ada, 1959), it was shown that addition of *N*-acetylmannosamine to the medium did not affect the growth of the organism (as estimated by counts of viable organisms).

Table 2. *Effect of adding* N-*acetylmannosamine, with and without an equimolecular amount of pyruvate, to the medium on the stimulation of neuraminidase production by* Vibrio cholerae

Enzyme titres in media inoculated with *Vibrio cholerae* were determined after 16 hr. incubation at 37°.

N-Acetyl-mannosamine concentration (μM)	With added pyruvate	No added pyruvate
	Enzyme titre	
2400	320	220
800	200	280
240	130	80
80	80	40
Nil	<10	<10

Effect of added pyruvate on the rate of neuraminidase production in medium containing N-*acetylmannosamine*

Addition of pyruvate (final concentration 600 or 3,000 μM) did not significantly affect the rate of production of neuraminidase in medium containing *N*-acetyl-mannosamine (600 μM). At the higher concentration, added pyruvate caused a noticeable increase in the growth rate of the organisms. The details of an experiment are plotted in Fig. 1.

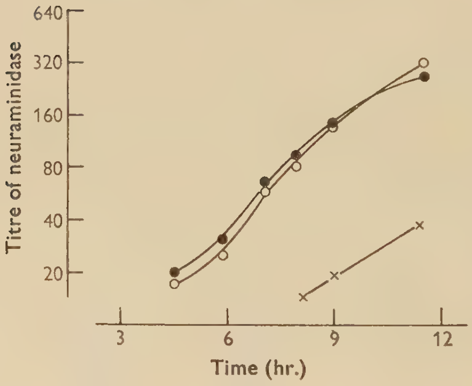

Fig. 1

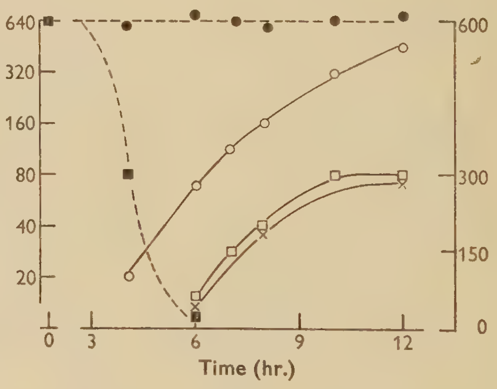

Fig. 2

Fig. 1. Effect of the addition of excess pyruvate on the rate of neuraminidase production in medium containing *N*-acetylmannosamine. Basal medium alone = ×—×; basal medium + *N*-acetylmannosamine (600 μM) = ○—○; basal medium + *N*-acetylmannosamine (600 μM) + pyruvate (3,000 μM) = ●—●.

Fig. 2. Rate of production of neuraminidase and the rate of disappearance of *N*-acetylmannosamine or *N*-acetylglucosamine added to media. Enzyme production in the presence of *N*-acetylmannosamine = ○—○; amount of *N*-acetylmannosamine present = ●—●. Enzyme production in the presence of *N*-acetylglucosamine = □—□; amount of *N*-acetylglucosamine present = ■- - -■. Enzyme production in basal medium = ×—×.

Metabolism of added N-*acetylmannosamine and* N-*acetylglucosamine during growth of the organisms*

N-Acetylmannosamine, when present in inoculated medium at 600 μM, persisted for at least 12 hr. incubation. On the other hand, *N*-acetylglucosamine in a similar concentration was rapidly metabolized. Details of an experiment are presented in Fig. 2. In a separate experiment, samples were removed at similar intervals, hydrolysed with acid and the total hexosamine content estimated. After correcting for the amount of hexosamine in the samples from the control medium, the following results were obtained. In the medium containing added *N*-acetylglucosamine, the value of the hexosamine reaction fell to the level of the control medium within 6 hr. incubation following inoculation. In the medium containing added *N*-acetylmannosamine, the value of the hexosamine reaction remained constant during 12 hr. incubation after inoculation.

Difference in the behaviour of N-*ANA and* N-*acetylmannosamine as a neuraminidase inducer*

The rates of production of neuraminidase by *Vibrio cholerae* in medium containing *N*-ANA (600 μM) and in medium containing *N*-acetylmannosamine (600 μM.) were found to differ. Details of an experiment which shows this difference in behaviour are plotted in Fig. 3.

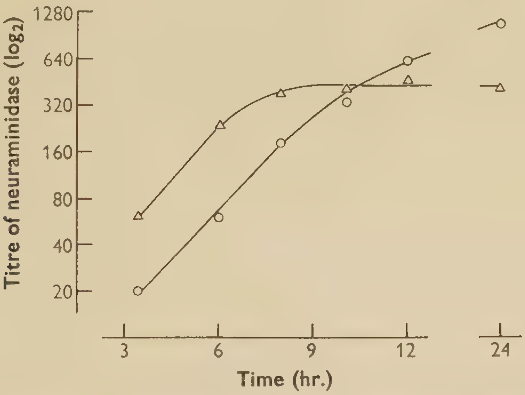

Fig. 3. Rate of production of neuraminidase in the presence of *N*-acetylmannosamine or *N*-acetylneuraminic acid. Basal medium + *N*-acetylmannosamine = ○—○; basal medium + *N*-acetylneuraminic acid = △—△.

Effect of added D-*mannosamine HCl on neuraminidase production*

Addition of D-mannosamine HCl (to 600 μM) to the basal medium severely inhibited bacterial growth as noticed visually and by counts of viable organisms. The production of neuraminidase by organisms grown in basal medium containing added D-mannosamine HCl was likewise profoundly affected. In a typical experiment, two portions of basal medium, one containing D-mannosamine HCl (600 μM), were inoculated to give a final count of $5{\cdot}4 \times 10^6$

organisms/ml. In the control tube the enzyme titre at 11 hr. was 100 and the viable count $9\cdot2 \times 10^8$ organisms/ml. In the tube containing D-mannosamine HCl, the enzyme titre at 12 hr. was 10 and the viable count $0\cdot35 \times 10^8$ organisms/ml. Estimation of the hexosamine content (no prior hydrolysis) of samples removed at different time intervals after inoculation (up to 12 hr.) showed that the added D-mannosamine persisted in the culture medium.

Effect of added glucosamine HCl *on neuraminidase production*

Addition of D-glucosamine HCl (to 600 μM) to the basal medium did not stimulate neuraminidase production. Estimation of the hexosamine content (no prior hydrolysis) of samples removed at different time intervals after inoculation (up to 12 hr.) indicated that the added D-glucosamine persisted in the culture medium.

Receptor gradient of neuraminidase produced in medium containing either N-*ANA or* N-*acetylmannosamine*

Burnet, McCrea & Stone (1946), and later Stone & Ada (1952), observed that when red cells, which are normally agglutinated by the strains of influenza virus used, were fractionally treated with neuraminidase, the agglutinability of these red cells by the viruses was progressively lost in an order which defines the so-called receptor gradient. It was found that treatment of red cells with neuraminidase, produced in our basal medium containing either *N*-ANA or *N*-acetylmannosamine or in soft nutrient agar, yielded red cells which exhibited the same receptor gradient. With fowl red cells the neuraminidase gradient obtained was: PR8, WSE, Mel and CAM, Swine, Lee, finally Mil. B. With human red cells the order was: WSE, PR8, Mel, CAM, Swine and Lee, Mil B.

DISCUSSION

N-acetylmannosamine induced the production of neuraminidase in *Vibrio cholerae* cultures, the amount of enzyme produced being approximately proportional to the concentration of inducer added. Addition of pyruvate to a medium containing *N*-acetylmannosamine did not appreciably affect the rate of production of the enzyme. This effect of *N*-acetylmannosamine was specific; two isomers—*N*-acetylglucosamine and *N*-acetylgalactosamine—were inactive. This latter finding confirms an earlier observation (Ada & French, 1957) that addition of *N*-acetylglucosamine and pyruvate to a peptone medium did not stimulate neuraminidase production by *V. cholerae*. The further observation was made that whereas *N*-acetylglucosamine added to the basal medium was readily metabolized by the organisms, the evidence obtained suggests that *N*-acetylmannosamine was not broken down. Thus the concentration of *N*-acetylhexosamine (or of total hexosamine after acid hydrolysis of the samples) in medium to which *N*-acetylmannosamine had been added remained the same during a period of 12 hr. incubation at 37° following inoculation. The present evidence suggests therefore that *N*-acetylmannosamine is a gratuitous inducer.

What then is the mechanism of neuraminidase induction by *N*-acetylmannosamine? It is helpful to point out the relationship of *N*-acetylmannosamine to the structure of a substrate of neuraminidase. This is shown in the accompanying diagram (Fig. 4) where *N*-ANA is shown linked to galactose as in sialyl lactose (see Gottschalk, 1957). Carbon atom 2 of the *N*-acetylhexosamine portion corresponds to C_5 of *N*-ANA in the diagram and is thus 4 atoms distant from the enzyme susceptible bond. What may be of great importance is the spatial disposition of the *N*-acetyl group to C_2 of *N*-ANA. Comb & Roseman (1958) showed that the partner with pyruvate in *N*-ANA is *N*-acetylmannosamine, not *N*-acetylglucosamine as previously thought. Our first reaction to the discovery that *N*-acetylmannosamine stimulated neuraminidase production was to consider the possibility that the *N*-acetylmannosamine was converted by the organism to *N*-ANA which was known to induce neuraminidase (French & Ada, 1959). The lack of effect of pyruvate when added to the system and the apparent resistance to enzymic attack of the added *N*-acetylmannosamine argued against this possibility. Conversely, the possibility was entertained that *N*-ANA might act as inducer by virtue of its degradation, by de-aldolization, into *N*-acetylmannosamine. At present two observations seem to argue against a quantitative conversion of *N*-ANA to *N*-acetylmannosamine. First, the breakdown of *N*-ANA in *Vibrio cholerae* cultures does not result in the accumulation of large amounts of an *N*-acetylhexosamine (French & Ada, 1959). Secondly, the finding that neuraminidase production stops as soon as *N*-ANA disappears from the medium would not be expected if *N*-acetylmannosamine were the sole degradation product, as can be seen from Fig. 3. In view of the findings of Comb & Roseman (1958) the above results were unexpected; clearly, more work needs to be done with our system. If the production of neuraminidase in medium containing added *N*-ANA ceases as soon as the intact *N*-ANA molecule disappears, this suggests a difference in the mode of action as inducer of *N*-ANA and *N*-acetylmannosamine. If, on the other hand, a degradation product of *N*-ANA is active as inducer, it is necessary to find the relationship between such a compound and *N*-acetylmannosamine. A further point which arises concerns the specificity of the enzyme produced on medium containing added *N*-ANA or on medium containing added *N*-acetylmannosamine. This aspect has not been fully investigated, but it has been shown that the two enzyme preparations acted

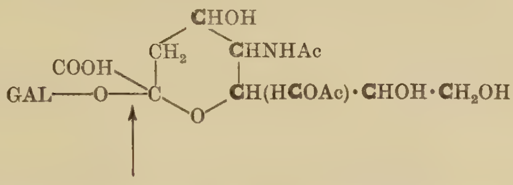

Fig. 4. Diagram showing the structure of the *N*-acetylneuraminic acid (*N*-ANA) portion of the sialyl lactose molecule. The carbon atoms of the *N*-ANA portion are numbered from left to right, no. 1 being the carbon atom of the COOH group. The carbon skeleton of the hexosamine portion of the *N*-ANA molecule is outlined in heavier print. The arrow indicates the bond split by neuraminidase.

on red cells in a similar fashion as evidenced by their identical receptor gradients.

Finally, the response of *Vibrio cholerae* to medium containing D-mannosamine or D-glucosamine is of interest. Both compounds appeared to resist enzymic attack by the organisms; D-mannosamine, but not D-glucosamine, severely interfered with the growth of the bacteria. No explanation is offered for these findings, but it is not unlikely that when better understood, the mode of action of *N*-acetylmannosamine as enzyme inducer may throw some light upon the mechanism of growth inhibition by D-mannosamine. It is quite clear that the 4Z strain of *V. cholerae* will be a useful tool in demonstrating some of the biochemical relationships of the amino sugars and their derivatives.

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Separation and Properties of Particles of Tobacco Rattle Virus with Different Lengths

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SUMMARY: Tobacco rattle virus was isolated from tobacco sap by differential centrifugation; the yield was about 50 mg. virus/l. sap. Purified preparations were highly infective, precipitated optimally at pH 4·0–4·5, and contained one electrophoretic component; chemical analysis and the ultraviolet absorption spectrum suggest they contain about 5 % nucleic acid and 95 % protein. Most rod-shaped particles in purified preparations were 73–77 mμ or 179–192 mμ long: all were 25 mμ wide. These two components were separated by rate zonal centrifugation in sucrose density-gradient solutions: both have the same density in solution as tobacco mosaic virus, and they have sedimentation constants of about 198s and 295s, respectively. No differences were found between long and short particles in serological behaviour, electrophoretic mobility or ultraviolet absorption spectrum; apparently they differ little in gross chemical composition. Only the long particles are infective. Particles slightly shorter than 179 mμ seem non-infective; some of these may be aggregates of two short ones. Short particles rarely aggregate end-to-end at pH 6·8–7·0 but aggregation increases as the pH value decreases.

Although tobacco rattle virus has been known for many years, occurs in many countries and has an extremely wide host range, only few of its intrinsic properties have been described. Among these, the rod-like shape (van der Want & Rozendaal, 1948; Paul & Bode, 1955), large temperature coefficient of thermal inactivation, stability in plant sap and failure to show photoreactivation (Cadman & Harrison, 1959), suggest similarities to tobacco mosaic virus; but the different dimensions of the virus particles and their lack of common antigens show that the two viruses are unrelated. The properties of purified preparations of tobacco rattle virus, described in the first part of this paper, give further evidence of similarity to tobacco mosaic virus. But unlike tobacco mosaic virus, and many other viruses with elongated particles, the lengths of particles of tobacco rattle virus fall into two major categories (Paul & Bode, 1955); the length of the longer particle is not a simple multiple of that of the shorter. The separation and properties of these two kinds of particle are described in the second part of this paper.

METHODS

The PRN isolate of tobacco rattle virus (TRV; Cadman & Harrison, 1959) was cultured in the inoculated leaves of White Burley tobacco (*Nicotiana tabacum* L.). Leaves with confluent lesions were macerated, the sap squeezed through muslin and stored at −20°. Infectivity was assayed by comparing the numbers of local lesions produced by different inocula in the primary leaves of French bean (*Phaseolus vulgaris* L. var. Prince) or in leaves of

Chenopodium amaranticolor Coste & Reyn. 'Super-floss Celite' powder (Johns-Manville Ltd.) was mixed with all inocula. Antiserum was produced by injecting a rabbit intravenously, initially with 3 mg. purified virus, and a month later with 4 mg.; the rabbit was bled 10 days after the second injection.

Electrophoretic measurements were made in a model 38A Perkin-Elmer apparatus with the Philpot-Svenson optical system, at a potential gradient of 9·5 V./cm.

For centrifugation in sucrose density-gradients (Brakke, 1953), plastic tubes of 50 × 12 mm. were used. Each density gradient was made from three sucrose solutions, usually 67, 134 and 200 g./l., but gradients made from 134, 200 and 267 g./l., 200, 267 and 334 g./l., or 267, 334 and 400 g./l., were also used: all sucrose solutions contained 0·033 M (pH 7·0) phosphate buffer and were stored at 5°. Density-gradient preparations were made by pipetting 1·5 ml. of the least dense solution into a tube, then introducing 1·5 ml. of the solution of intermediate density at the bottom of the tube by using a Pasteur pipette, and finally, 1·0 ml. of the densest solution was similarly introduced below the other two. The density-gradient preparations were kept overnight at 5° before centrifuging in the no. 4 swing-out head of an MSE 'Super-speed 25' refrigerated centrifuge. Both centrifuge rotor and virus preparation were also cooled to 5° before use; immediately before centrifugation, 0·4–0·5 ml. virus preparation was floated on each density-gradient mixture. After centrifugation the temperature of the liquid was 5–8°. At 16,000 rev./min., the speed usually used, the centrifugal force was 20,400 ***g*** at the top of the column of liquid and 31,700 ***g*** at the bottom. The centrifuged density-gradient preparations were viewed in a vertical beam of light against a dark background and samples of 0·2–0·4 ml. removed by carefully lowering to the required depth a Pasteur pipette whose tip was bent through an angle of 90°. The samples were dialysed overnight at room temperature against 0·1 M-ammonium acetate at pH 6·8, and assayed for infectivity and particle content.

The concentration of virus particles was estimated by a spray-droplet technique (Nixon & Fisher, 1958). One vol. dialysed sample was mixed with 1 vol. 0·1 % bovine serum albumin and 1 vol. standard latex suspension (Dow Chemical Co.; particles designated 88 mμ diameter by the makers). The mixture was sprayed on to electron microscope grids, which were examined with a Siemens Elmiskop I microscope and the different types of particles in the droplet traces counted on the fluorescent screen. Estimates of virus content made by the spray-droplet method, when converted to mg./l., were only 78 % of the values indicated by dry-weight determinations or calculated from nitrogen analyses. The difference probably resulted from an overestimate of the mean diameter, and hence the weight, of the latex particles in the standard suspension (Nixon & Fisher, 1958). No allowance for this discrepancy was made in the counts of virus particles given below.

RESULTS

Method of concentrating tobacco rattle virus

Tobacco rattle virus is stable and readily concentrated; differential centrifugation was the method finally adopted. Infective tobacco sap, stored at −20° for at least a month, was thawed and centrifuged for 3 min. at 9000 *g*, when the clear supernatant fluid was decanted and centrifuged for 90 min. at 75000 *g*. The resulting pellet was resuspended in pH 7·5 borate buffer or pH 6·4 citrate buffer of one-tenth the original volume. After this suspension had been taken through a second cycle of low- and high-speed centrifugation, the translucent faintly coloured pellet was resuspended in pH 6·4 citrate buffer of one-twentieth the original volume of sap and centrifuged at low speed. The supernatant fluid was kept at 20° for 2–3 days before centrifuging for 120 min. at 57000 *g*; the colourless translucent pellet was resuspended in citrate buffer, centrifuged at low speed, and the resulting supernatant fluid was the final preparation. The final preparations contained 0·5–2·0 mg. virus/ml., indicating a virus concentration in sap of about 50 mg./l.; these are referred to below as 'purified preparations'. When needed for injecting rabbits, pellets from the last high-speed centrifugation were suspended in 0·85 % sodium chloride solution; preparations used for density-gradient centrifugation were made by only two cycles of high- and low-speed centrifugation and the final pellets were suspended in 0·033 M-phosphate buffer (pH 7·0).

Properties of purified preparations of tobacco rattle virus

Purified preparations were slightly opalescent at 100 mg./l. but even at 2000 mg./l. solutions did not show anisotropy of flow when rocked in narrow tubes between crossed polarizers; they were infective when diluted to 0·01 mg./l. but not at 0·001 mg./l. With virus antiserum, they precipitated specifically down to 2–5 mg./l.; there was only one optimal precipitation zone. They did not precipitate with tobacco mosaic virus antiserum, and tobacco mosaic virus did not precipitate with tobacco rattle virus antiserum.

Electrophoretic behaviour. When examined electrophoretically, preparations containing 1 mg. virus/ml. showed one tall slender peak, with a mobility of $-1{\cdot}7 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ V.$^{-1}$ in 0·067 M-phosphate buffer (pH 7·0), only a fifth of the value for tobacco mosaic virus under the same conditions. After electrophoresis had gone on for an hour, there was still only one peak, but it had broadened more than the peak given by tobacco mosaic virus, presumably because tobacco rattle virus diffuses more rapidly.

Chemical composition. Chemical analyses were made by Miss M. Byers and Dr W. S. Pierpoint. Nitrogen was determined by a micro-Kjeldahl method, total phosphorus by the method described by Holden & Pirie (1955), and carbohydrate (using a ribose standard) by an orcin method. The purified preparations contained about 16·5 % N, 0·42 % P and 2·8 % carbohydrate, values which are consistent with the composition of a nucleoprotein containing 5 % nucleic acid and 95 % protein.

Ultraviolet absorption spectrum. The ultraviolet absorption curve of tobacco rattle virus closely resembled that of tobacco mosaic virus.

Precipitation in acid solutions. Like tobacco mosaic virus, tobacco rattle virus precipitates when virus preparations are acidified. Thus, when samples of a tobacco rattle virus preparation were suspended in citrate buffers of various pH values, precipitation was heaviest at pH 4·0–4·5 (Table 1). The precipitate produced at pH 4·5 was infective, though less so than control preparations not acidified. At pH values below 4·5 the particles were disrupted and no rods were found by electron microscopy in samples adjusted to pH 3·8. The infectivity of the virus in sap is similarly affected by acid (Cadman & Harrison, 1959). Tobacco rattle virus precipitates when dialysed against distilled water of pH 4·5, a value reached when carbon dioxide is absorbed from the air during storage: the precipitates dissolve when dialysed against freshly boiled water, with a pH of 6·5 –7·0.

Table 1. *Effect of* pH *value on precipitation of tobacco rattle virus*

Samples (0·1 ml.) of a purified virus preparation were mixed with 0·4 ml. citric acid/sodium hydroxide/hydrochloric acid buffer solutions. The mixtures were observed for 30 min., then centrifuged for 3 min. at 9000 *g*, and the infectivity of the supernatant fluids and resuspended sediments assayed after readjusting all samples to pH 7·0 and 2·5 ml.

	Infectivity* of		
pH	Supernatant fluid	Resuspended sediment	Amount of precipitation
2·0	0	0	Trace
3·0	0	0·6	Slight
4·0	0·2	1	Great (small floccules)
4·5	13	160	Great (larger floccules)
5·0	326	45	Moderate
7·0	1000	192	0

* Assayed on French bean; figures are relative to supernatant fluid at pH 7·0 (= 1000).

Electron microscopy. Purified tobacco rattle virus preparations contain rod-shaped particles of various lengths, which when viewed end-on appear to have a hollow centre (Pl. 1, fig. 2). All lengths from 25 to 200 mμ, occur, but the lengths of most particles fall into two categories (Fig. 1). Critical measurements, made by examining electron micrographs with an optical microscope, showed the two categories to be 73–77 mμ and 179–192 mμ respectively. The average values, 75 and 185 mμ, agree well with those of 70 and 180 mμ given by Paul & Bode (1955), and confirm that the length of the longer particles is not a simple multiple of that of the shorter ones. Figure 1 also shows that the proportions of particles of different lengths originally present in frozen sap were not altered much by the purification processes. The proportions of particles of different lengths were the same when preparations were suspended in pH 7·0–8·0 borate or phosphate buffers, or in pH 6·8 ammonium acetate. The width of all rods was about 25 mμ, but great accuracy cannot be claimed for measurements of this magnitude made on shadowed preparations. If the density of tobacco rattle virus is assumed to be 1·33, the average value given for tobacco mosaic virus by Bawden & Pirie (1937; the density of tobacco rattle virus in solution is the same as that of tobacco mosaic virus), the longer particles have an average mass equivalent to a molecular weight of $72 \cdot 7 \times 10^6$, and the shorter particles of $29 \cdot 5 \times 10^6$.

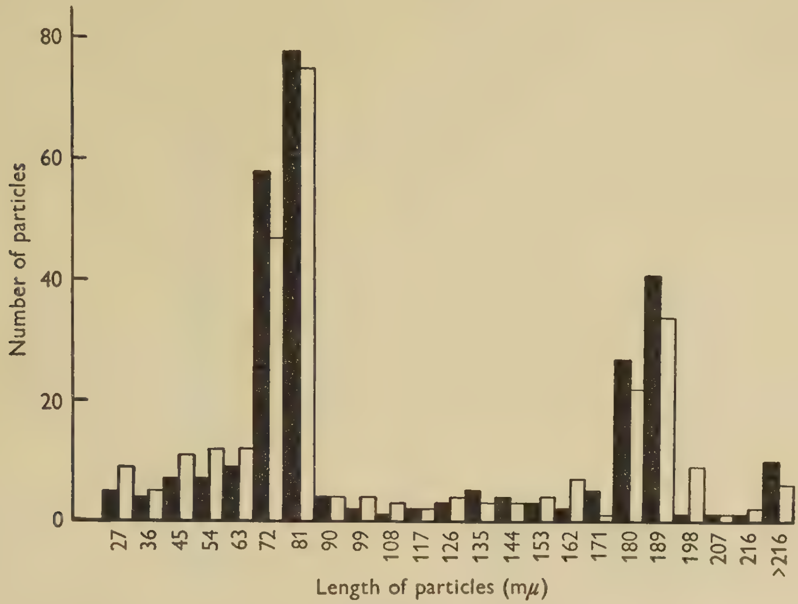

Fig. 1. Proportions of tobacco rattle virus particles of different lengths in tobacco sap after freezing (black columns) and after subsequent purification (white columns). Particle lengths are divided into categories of 9 mμ.

Separation and properties of long and short particles

The longer particles of tobacco rattle virus are about 2·5 times as long as the shorter ones and are clearly not aggregates of them. It therefore seemed of interest to separate the two kinds of particles and compare their properties.

Separation and infectivity of long and short particles. Differential ultracentrifugation did not give a useful fractionation, but centrifuging purified preparations of tobacco rattle virus in sucrose density-gradients produced two opalescent zones. The top of the upper one was clearly defined, but the top of the lower one was not; the upper zone was usually broader than the lower, and both were narrower than the virus preparation initially floated on the density-gradient (Pl. 1, fig. 1). Table 2 shows the infectivity and the number of long and short particles in samples from above, below, and the middle of, each zone. For convenience, particles twice or more as long as the commonest type of short particles were counted as 'long particles' and the rest as 'short particles'. Short particles were most concentrated in the top zone and long particles were mostly in the bottom zone. The relative thicknesses and amounts of opalescence of the two zones therefore depended on the ratio of long to short particles in the unfractionated purified preparation. This ratio has varied from 1:2 to 1:18 in different preparations; the cause of the variations is unknown. Thus, whereas the top zone was always obvious, the bottom zone was often too indistinct for its depth to be measured accurately.

Table 2 also shows that specific infectivity increases with depth of sampling and that the relative infectivity of the samples was proportional to the number of long particles they contained. Indeed, the ratio of the concentration of long particles to relative infectivity was remarkably constant, and the similarity of the ratios for the samples from the density-gradients to those for the unfractionated preparations shows that little or no infectivity was lost during the centrifugation procedures. The relative infectivity of the samples was the same for tobacco or *Chenopodium amaranticolor* as for French bean. The short particles seemed to be non-infective.

Table 2. *Separation of two components of tobacco rattle virus preparations by centrifuging in a sucrose density-gradient*

Purified virus preparation (0·4 ml.) was floated on density-gradients made from 1·5 ml. 67 g./l., 1·5 ml. 134 g./l. and 1·0 ml. 200 g./l. sucrose solutions. After 2 hr. centrifugation at 16,000–17,000 rev./min., samples were removed, diluted 1/4 with water, dialysed overnight against 0·1 M-ammonium acetate and assayed for infectivity and particle content. Unfractionated samples, diluted 1/10 with water, were also dialysed and assayed.

Depth sampled (mm.)	Virus particles/ml. ($\times 10^8$)*		Relative infectivity†	Ratio of no. long particles ($\times 10^8$): relative infectivity	Specific infectivity (lesions/μg. virus)
	Long	Short			
		Expt. 1 (16,000 rev./min.)			
12–15	0	4	0	—	0
17–19‡	0	7,600	1	—	0·04
21–23	739	3,200	597	1·2	34·0
24–26‡	1,920	1,080	1,183	1·6	58·0
28–30	368	96	302	1·2	86·0
Unfractionated	2,720	7,600	1,770	1·5	39·0
		Expt. 2 (17,000 rev./min.)			
14–17	5	11	1	5·0	12·0
20–22‡	173	4,740	2	86·5	0·1
23–25	110	5,040	6	18·3	0·3
26–28‡	2,300	1,540	996	2·3	39·0
30–32	1,540	685	620	2·5	40·0
Unfractionated	3,650	10,960	1,611	2·3	23·0

* 'Long' particles were $>$ 140 mμ; 'short' particles $<$ 140 mμ in length.
† Total lesions on eight half-leaves of French bean.
‡ Position of visible zones.

The long and short particles were not completely separated in one centrifugation, and could be fractionated further by again centrifuging either top or bottom zones (Table 3). After the second centrifugation, samples from the top zone were almost non-infective, and contained very few long particles (Fig. 2; Pl. 1, fig. 4). Moreover, of those counted as 'long' in spray-droplet assays, most were in the range 140–170 mμ. There is no evidence, however, that particles 140–170 mμ in length were infective, and indeed they were probably not, for samples in which they formed a large proportion of the long particles counted were much less infective than samples containing a similar number of particles over 170 mμ in length. Examples of samples containing particles of 140–170 mμ are those from 20–22 mm. and 23–25 mm. in Expt. 2,

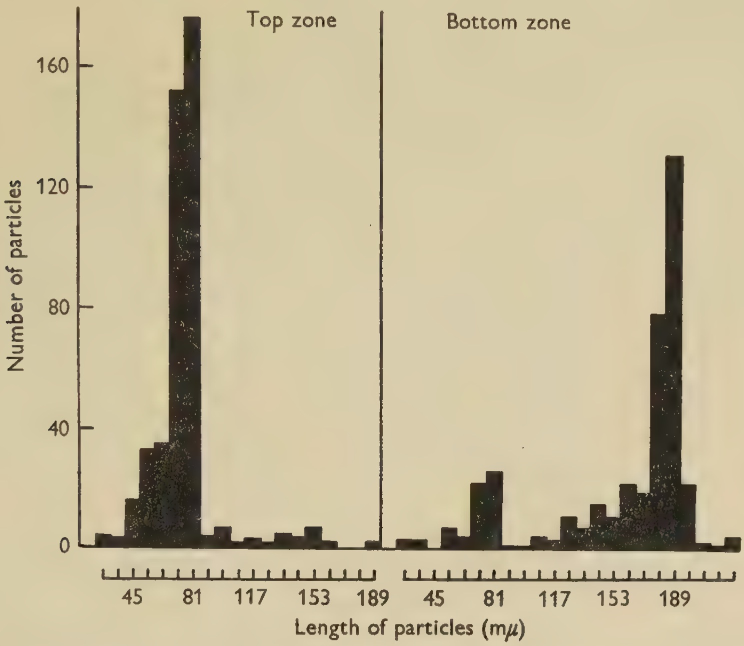

Fig. 2. Proportions of tobacco rattle virus particles of different lengths in a sample from (left) the top, and (right) the bottom opalescent zone after two centrifugations in a sucrose density-gradient. Particle lengths are divided into categories of 9 mμ. The last column in the right-hand diagram represents all particles longer than 216 mμ.

Table 3. *Separation of infective and non-infective particles of tobacco rattle virus by two cycles of centrifugation in a sucrose density-gradient*

Preparation of density-gradients, centrifugation and assays were done as described in Table 2.

Sample from	Relative infectivity	Virus content (mg./l.)	Specific infectivity (lesions/μg. virus)
Top zone, first gradient	9·5	192	0·05
Top zone, second gradient	0·5	36	0·014
Bottom zone, first gradient	1840	103	17·9
Bottom zone, second gradient	881	36	24·5
Unfractionated preparation, diluted 1/10	898	111	8·1

Table 2; the possibility that these particles were aggregates of two short ones is discussed further below.

Although preparations containing only short particles were obtained by recentrifuging samples from the top zone, preparations of long particles always contained some short ones (Fig. 2; Pl. 1, fig. 3). This was probably because a few long particles, perhaps initially aggregates of short ones, fragmented during or after centrifugation.

Sedimentation rates. Using Brakke's (1958) methods and tobacco mosaic virus as a standard, the sedimentation rates of the two common kinds of tobacco rattle virus particles were estimated. In the first method, density-gradients were made from solutions of 67, 134 and 200 g. sucrose/l.; purified tobacco rattle virus was added to some tubes and purified tobacco mosaic virus to others. The tubes were then centrifuged for different periods at 16,000 rev./min. and the depths of the tops of the opalescent zones measured. After applying a correction for the times taken by the centrifuge to accelerate and decelerate, the lines obtained by plotting depth of zone against time were almost straight, both with tobacco mosaic virus and with each tobacco rattle virus zone. The times taken to reach an arbitrary depth (15 mm.) were determined graphically and, assuming their reciprocals to be in the ratios of their sedimentation constants (Brakke, 1958), particles in the top zone of tobacco rattle virus were calculated to have a constant 1·06 times, and those in the bottom zone 1·66 times, that of tobacco mosaic virus.

This method works well only when the density of the viruses compared is the same. In the second method, both densities and sedimentation constants were determined. The sedimentation rates of the tobacco rattle virus zones were compared with that of tobacco mosaic virus in sucrose gradients covering a wide range of densities. Using these results and the formula given by Brakke (1958), and assuming the density of tobacco mosaic virus in solution to be 1·27 (Schachman & Lauffer, 1949), Fig. 3 was plotted. Although there were fewer reliable points for the bottom zone than for the top, Fig. 3 shows that the particles in each zone had a density of about 1·27, the same as that of tobacco mosaic virus. From the intercepts at $\rho' - \rho = 0$, the sedimentation constants of the particles in the two tobacco rattle virus zones were given as 1·06 and 1·58 times that of tobacco mosaic virus. If the latter value is taken to be 187 s (Lauffer, 1944), the values for the long and short particles of tobacco rattle virus are 295 s and 198 s, respectively.

Serological tests and ultraviolet absorption spectra. Table 4 shows some properties of a sample from the top zone of a density gradient and of one from a recentrifuged bottom-zone: in the first sample, 94 % by weight of the material was short particles, and in the second, 88 % was long particles. The concentration in both samples was similar and both precipitated to similar end-points with antiserum to unfractionated tobacco rattle virus. The serological precipitates formed by the two samples appeared to be very similar, and were of the somatic type. The ultraviolet absorption spectra (230–300 mμ) of the two samples were almost identical, evidence that both long and short particles are nucleoproteins of similar gross composition.

In further experiments on the antigenic constitution of long and short particles, gel-diffusion tests were made on slides (Mansi, 1958). Preparations of short particles and unfractionated virus preparations each produced one line of precipitation with virus antiserum, and the two lines joined. Absorption

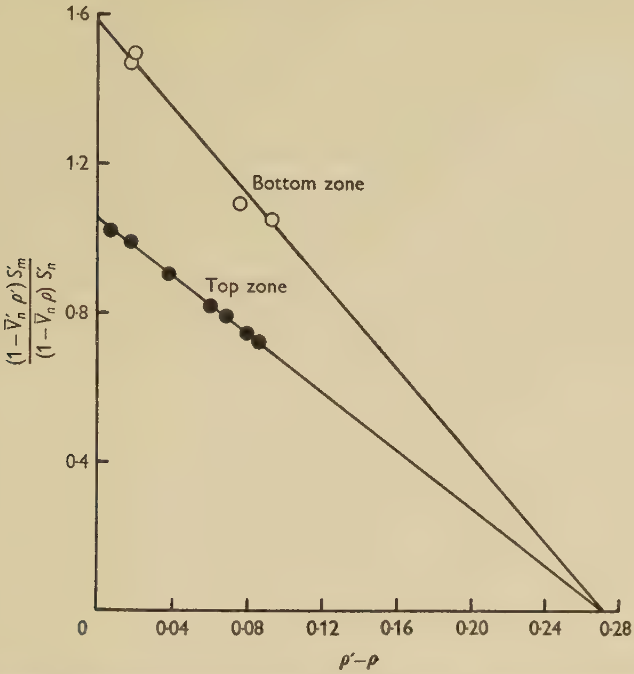

Fig. 3. Diagram of the sedimentation rates of top and bottom zones of tobacco rattle virus in sucrose density-gradients relative to that of tobacco mosaic virus. $\bar{V}_n = \bar{V}'_n$ = partial specific volume of tobacco mosaic virus; S'_m/S'_n = ratio of the sedimentation rate of the tobacco rattle virus zone to that of tobacco mosaic virus; ρ = density of water at 20°; ρ' = density of sucrose solution at half the depth reached by the tobacco rattle virus zone.

Table 4. *Properties of particles of tobacco rattle virus from top and bottom zones of a sucrose density-gradient*

Preparation of density-gradients, centrifugation, infectivity tests and particle counts were done as indicated in Table 2.

	Sample from	
	Top zone	Bottom zone
Depth (mm.)	15–17	21–23
Long particles/ml. (× 10^8)	158	2400
Short particles/ml. (× 10^8)	6560	816
Relative infectivity*	23	462
Ratio of no. long particles (× 10^8): relative infectivity	6·9	5·2
$E260/E280$	1·11	1·10
Serological precipitation end-point	1/16	1/8
Virus content (mg./l.)	43·2	42·3

*Assayed on *Chenopodium amaranticolor*.

of antiserum with short particles abolished its ability to form a line with either short particles or unfractionated preparations. Preparations containing predominantly long particles did not form a line with antiserum, either because they were too dilute or because long particles diffused through 1 % agar gel too slowly. These tests did not reveal antigenic heterogeneity, but neither did they show that long and short particles contained the same antigens. Precipitation tests in tubes were therefore made with unfractionated preparations and with samples from twice-centrifuged top and bottom zones. Ninety-seven % of the virus in sample 'top 2' was short particles and 95 % of that in sample 'bottom 2' was long particles (Table 5). Of the 3 % long particles in sample 'top 2', a large proportion were non-infective and shorter than 170 mμ; many were probably aggregates of two short particles, so their presence does not complicate interpretation of the results. The antiserum, prepared against an unfractionated preparation, had the same titre against all three antigens, and when partially absorbed with short particles, it still had a similar, but much lower, titre against all three (Table 5). Long and short particles, therefore, have few or no antigens not possessed by each other.

Table 5. *Serological behaviour of particles of tobacco rattle virus from top and bottom zones of sucrose density-gradients*

Preparation of density-gradients, centrifugation and assays were done as indicated in Table 2.

(*a*) *Composition of antigen preparations*

Antigen preparation	Virus particles/ml. ($\times 10^8$) Long	Short	Relative infectivity	Virus concentration (mg./l.)	Specific infectivity (lesions/ μg. virus)
Top 2	208	17,400	3	110	0·03
Bottom 2	647	81	652	10·3	63·2
Unfractionated, diluted 1/4	3,320	16,600	10,600	154	68·8

(*b*) *Precipitation end-points* of antiserum*

Antigen preparation	Antiserum† Untreated	Absorbed with 9 vol. top 2
Top 2, diluted 1/2	1/160	1/10
Bottom 2, undiluted	1/160	1/10
Unfractionated, diluted 1/8	1/160	1/20

* Recorded after 5 hr. incubation at 38°.

† Prepared against an unfractionated virus preparation.

Aggregation of short particles. In contrast to many other viruses with elongated particles, tobacco rattle virus particles do not aggregate end-to-end much at pH 7. Indeed, it is their failure to aggregate which allows the separation of long and short particles in sucrose density-gradients. However, as already noted, considerable numbers of particles 140–170 mμ long were sometimes present in samples from the top zone of sucrose density-gradients.

In one experiment, the proportion of such particles in a sample from the top zone increased from 16 to 59 % of the total weight of virus present during 14 days' storage at pH 6·5 and 5°, and as the total virus content of the sample was the same at the beginning and end of the storage period, the additional medium-length particles seem to have formed by aggregation of smaller ones.

To see whether such aggregation depends on pH value, portions of a sample from the top zone were dialysed against ammonium acetate/acetic acid/ammonia mixtures of pH 5·6, 6·4 and 7·2; respectively, the resulting preparations each had a trace of infectivity. Examination of droplet traces showed that nearly all the particles were of three kinds, short, medium length (140–170 mμ) and very long (200–750 mμ) types. Very long forms were common at pH 5·6, occasional at pH 6·4 and did not occur at pH 7·2. Medium length rods accounted for 35–50 % of the total weight of virus present in all three preparations, whereas typical short particles increased from 17 % at pH 5·6 to 53 % of the virus at pH 7·2. Thus, aggregation increased with decrease of pH value. The previous experiment showed that small particles aggregated to form ones of medium length; the present experiment suggests that both these types aggregate further to produce very long forms at pH 5·6–6·4. Whether all medium-length particles are formed by aggregation of two short ones is unknown; some medium-length particles were stable at pH 7·2, and some were still present after the last traces of sucrose were removed by prolonged dialysis at pH 6·8.

DISCUSSION

It is extremely difficult to draw valid conclusions from measurements of the lengths of virus particles (Pirie, 1957). Nevertheless, our results are probably free from some of the commoner sources of error. First, tobacco rattle virus particles rarely aggregate when preparations are kept for short periods at pH 7. Secondly, the common occurrence of particles of two different lengths separated by a factor of 2·5 can hardly arise from selective extraction methods, as can be argued with the many other viruses which have only one common length. Thirdly, different methods of extracting tobacco rattle virus from plants give similar results; Paul & Bode (1955) found in leaf exudate and in fresh clarified sap from different species a similar range of particle lengths, and similar proportions of long and short particles to those we describe. We therefore conclude that tobacco rattle virus has two characteristic lengths which probably occur together in the plant. By analogy, results with tobacco rattle virus support the idea that the most common, or 'normal', lengths of several other viruses in leaf exudates (e.g. Brandes & Paul, 1957) are intrinsic properties of the viruses, are little affected by the host species and do not depend on any particular method of preparing plant extracts for electron microscopy.

Tobacco rattle virus is unusual in that the lengths of the two common types of particle differ by a factor of 2·5. Our admittedly crude tests suggest that the two types have a similar gross chemical composition, for they have the same ultraviolet absorption spectrum, the same density in solution, and mixtures of particles of the two types showed only one peak when examined electrophoretically and only one optimal zone of precipitation with virus antiserum. Also, absorption of antiserum with short particles abolished its ability to precipitate long ones. Whether the small particles are precursors or breakdown products of the larger ones, or are additional products of virus-infected cells, is unknown; but the relationship between them may not be unique among plant viruses, because purified preparations of the Rothamsted culture of tobacco necrosis virus contain nucleoprotein spheres of two sizes, and whereas the smaller particles are non-infective, infective preparations always contain the larger ones (Bawden & Pirie, 1950).

There is some direct evidence with wheat streak mosaic virus (Brakke & Staples, 1958) and much indirect evidence with tobacco mosaic virus (e.g. Bawden & Pirie, 1945) that the 'normal' length is the minimum infective length. Although short particles are the most abundant ones in purified preparations of tobacco rattle virus, and they seem non-infective, our results do provide strong evidence for a minimum infective length, which is also a common one. Indeed, tobacco rattle virus particles only slightly shorter than 179 mμ have little or no infectivity, and the trace of infectivity in some preparations of short particles is readily explained by the observed contamination with a few particles 179–192 mμ long. It might be argued that a minute proportion of short particles is infective or that many together can infect although each alone cannot, but such arguments seem unnecessary. We have no idea what proportion of the long particles is infective; if single particles can infect, one in 100,000 would be enough to explain the observed results.

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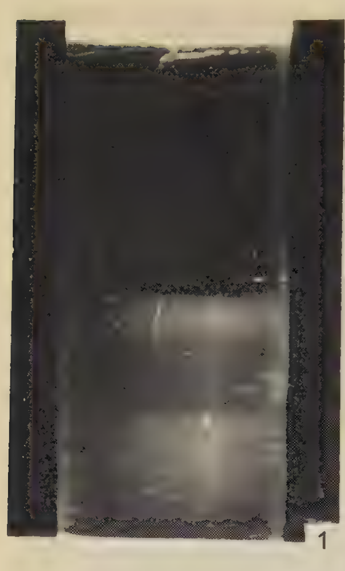

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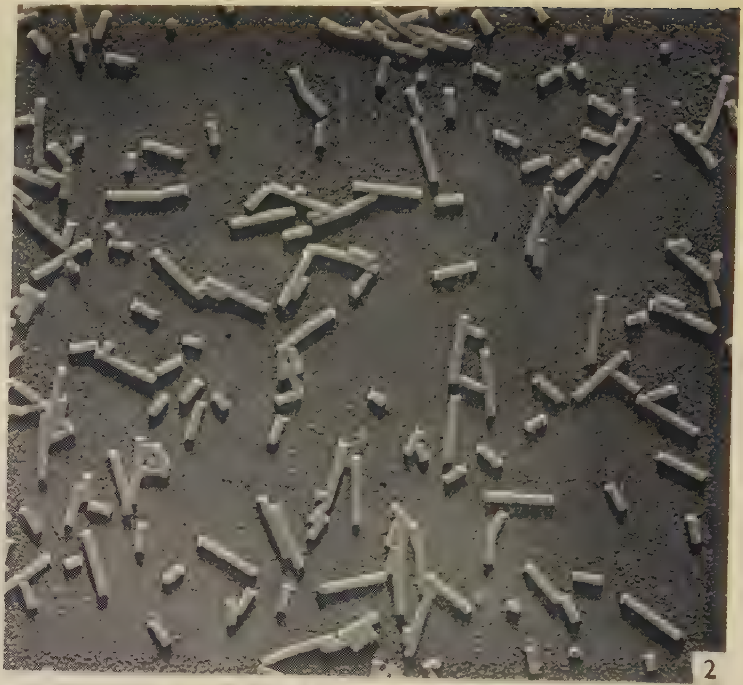

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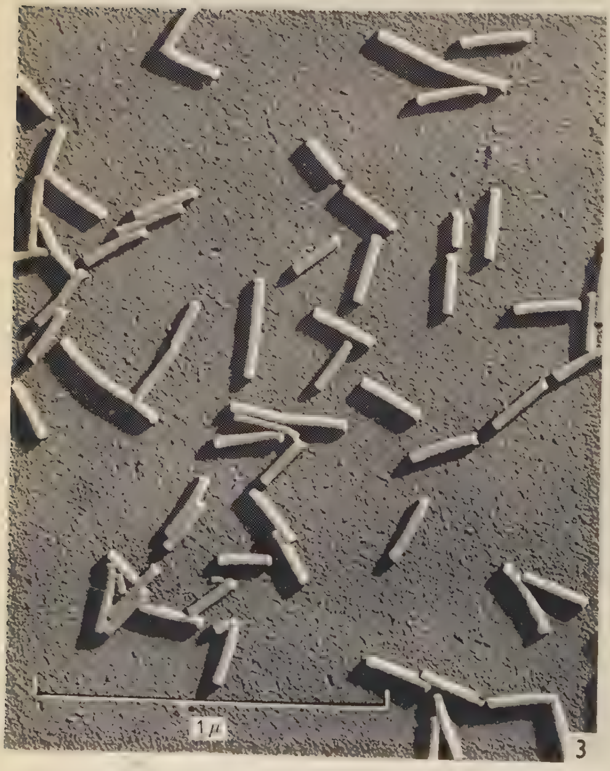

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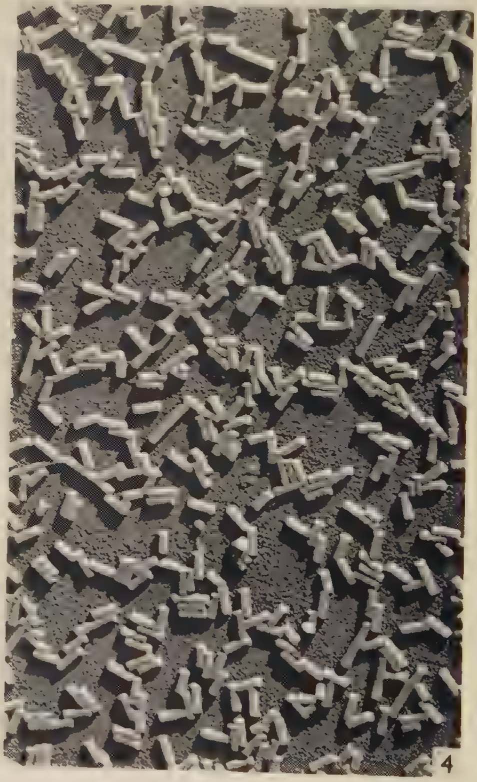

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B. D. Harrison & H. L. Nixon—Fractionation of tobacco rattle virus.
Plate 1

(*Facing p.* 581)

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EXPLANATION OF PLATE

Fig. 1. Enlarged view of a centrifuge tube after centrifuging tobacco rattle virus for 100 min. at 16,500 rev./min. in a density-gradient made from 67, 134 and 200 g./l. sucrose solutions. Note the two opalescent zones.

Fig. 2. Electron micrograph of a purified but unfractionated preparation of tobacco rattle virus. A few particles are seen end-on. × 46,200.

Fig. 3. Electron micrograph of particles from the bottom opalescent zone after two centrifugations in a sucrose density-gradient. × 46,200.

Fig. 4. Electron micrograph of particles from the top opalescent zone after two centrifugations in a sucrose density-gradient. × 46,200.

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Electron Microscopic Evidence on the Structure of the Particles of Tobacco Rattle Virus

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SUMMARY: The structure of tobacco rattle virus was studied by examining with the electron microscope: (1) shadow-cast mounts of particles partly degraded with alkali, sodium dodecyl sulphate or phenol; (2) unshadowed mounts on thin carbon film of particles treated with solutions of lanthanum nitrate, uranyl acetate, osmium tetroxide, phosphomolybdic acid or phosphotungstic acid.

The particles were tubular, with a central hole of approximately 4 mμ diameter and an outside diameter which varied from 17 to 25 mμ according to treatment. Next to the central hole, which could be filled with lanthanum nitrate or with uranyl acetate, was a region 1–1·5 mμ thick, which stained heavily with osmium tetroxide, phosphomolybdic and phosphotungstic acids. The rest of the particle stained lightly with uranyl acetate, phosphomolybdic and phosphotungstic acids, and showed transverse bands 2·5 mμ apart. It is suggested that these bands may represent a helical structure similar to that of tobacco mosaic virus, which tobacco rattle virus resembles in many respects.

Some properties of tobacco rattle virus have already been reported (Harrison & Nixon, 1959) and we now describe the structure of the particles as revealed by electron microscopy.

METHODS

The preparations of tobacco rattle virus used were made as already described (Harrison & Nixon, 1959). For shadow-cast mounts the suspensions were usually mounted as gross drops on copper grids bearing collodion films. These were then shadowed with platinum in a modified commercial evaporating plant, using a pumping time of at least 2 hr. to minimize the grain size of the deposit. About 0·8–1·0 mμ of platinum was usually deposited on the grids, which were placed 6–8 cm. from the apex of a V-shaped tungsten filament made from two strands of 0·5 mm. wire twisted together. Stained particles were mounted, usually as gross drops, on carbon films made by direct stripping from glass or freshly cleaved muscovite mica, and picked up on 4- or 7-hole platinum disks. Sometimes the suspensions were sprayed on to the mounts with the 'aerograph' air brush used for spraying suspensions in particle counting work (Nixon & Fisher, 1958).

The following reagents were used as stains:

Lanthanum nitrate: 2% (w/v) in water; pH *c.* 4·0.
Osmium tetroxide: 2% (w/v) in water; pH *c.* 5·0.
Phosphotungstic acid: $P_2O_5.24WO_3.xH_2O$ 0·01–5·0% (w/v) in water; 2% solution was pH 2·3.
Phosphomolybdic acid: $P_2O_5.24MoO_3.xH_2O$ 0·01–5·0% (w/v) in water; 2% solution was pH 2·0.
Uranyl acetate: 2% (w/v) in water; pH *c.* 4·2.

Phosphotungstic and phosphomolybdic acids were also used as 1% (w/v) solutions adjusted to pH 6·5 with NaOH (Brenner & Horne, 1959). All these 'stains' except osmium tetroxide contain metals which give insoluble phosphates, and it was therefore necessary to free the virus suspensions from phosphate by dialysis against water or 0·1 M-ammonium acetate solution when, as often happened, a phosphate buffer had been used as the final suspending fluid during virus purification. For the staining treatments 0·1–0·5 mg. virus in 0·1–0·5 ml. was pipetted into a centrifuge tube and stain added in excess (1–4 ml.). After shaking, the tubes were allowed to stand for about 1 hr. before centrifuging to pack the virus, which was invariably precipitated by the treatment. The pellets were resuspended in distilled water or 0·1 M-ammonium acetate and appropriately diluted for mounting as already described. Residual ammonium acetate, when present, was volatilized by gently heating the mount on a molybdenum strip in the evaporating plant before examination in the microscope. Attempts to evaporate the ammonium acetate with the electron beam in the microscope invariably broke the films.

Enzyme treatments. Virus solutions were incubated for 1 hr. at 37° in M/15 phosphate buffer (pH 7·0) containing either crystallized trypsin (Armour Pharmaceutical Co.) at 1 mg./l., crude trypsin at 500 mg./l., or crystallized ribonuclease (Armour Pharmaceutical Co.) at 1 mg./l.

Electron microscopy. All the preparations were examined in a Siemens Elmiskop I electron microscope, with a 50 μ objective aperture and fine focus condenser. Normally the microscope was operated at 80 kV, but 60 kV was used when maximum contrast was necessary. Shadowed preparations were usually photographed at × 20,000 and stained ones at × 80,000; with stained ones, three or more photographs were usually taken in a through focus series, and the best photograph selected for further photographic enlargement. Measurements of the structures described below were made from prints at final magnifications of × 150,000–500,000; the microscope was calibrated by the two-stage procedure recommended by the makers, with a 70 μ hole in a platinum disk, measured with a calibrated light microscope, as a primary standard.

RESULTS

Shadowed particles of purified virus

Particle lengths of tobacco rattle virus have already been discussed (Harrison & Nixon, 1959) and will not be further considered here. The particles in shadowed mounts seemed to be *c.* 25 mμ wide when they lay with their long axes in the direction of shadowing. Particle widths will be further considered below in the section on stained particles. The ends of a few particles appeared collapsed, and this suggestion of a tubular structure was supported by the ring-like appearance of very short particles which had dried end-on to the supporting film (Pl. 1, fig. 1). These rings had a central hole about 4 mμ in diameter, and were rather similar to the end-on views of tobacco mosaic virus fragments obtained by Williams (1952) after ultrasonic treatment. Williams, however, obtained some tobacco mosaic virus rings with a hexagonal outline when the mounts had been made from freshly-treated virus preparations. We have not observed these with tobacco rattle virus, and our failure to do so can hardly be due to lack of resolving power, for tobacco rattle virus is larger than tobacco mosaic virus and our instrument normally has a resolving power of *c.* 1 mμ.

Partial degradation followed by examination of shadowed material. Alkali and sodium dodecyl sulphate partly degrade tobacco mosaic virus (Sreenivasaya & Pirie, 1938; Schramm, 1947; Hart, 1955), and these treatments were therefore applied to tobacco rattle virus to see whether they would produce fragments which might give information about the structure of the particles. The most useful alkali treatment was to heat virus suspension in 0·05 M-sodium carbonate + borate buffer (pH 10·2). Virus heated under these conditions to 40° for 12 min. retained some infectivity, but not after heating to 45° for 12 min. Of the few particles found with the electron microscope in such preparations most were normal in appearance, but occasionally one was found with a structure suggesting that the outer part of the particle had been partly stripped from a core, which appeared to be about 15 mμ in diameter (Pl. 1, fig. 3). Rings were much more numerous in the treated virus preparations than in the untreated ones; they had holes about 6 mμ in diameter (Pl. 1, fig. 4) larger than those in untreated preparations, but smaller than the core of the particle shown in Pl. 1, fig. 3, and referred to above. Shadow lengths suggest that these rings with large holes may be thinner than those in untreated preparations (Pl. 1, fig. 1), but it is difficult to be certain of this because preparations of partly-degraded particles contain much low-molecular weight material which obstructs observations and measurements. Very similar pictures were obtained after treatment with 0·02 % (w/v) sodium dodecyl sulphate at pH 7·0 for about 1 min. at 60°, or for about 5 min. at 50° (Pl. 1, fig. 2). Such preparations had lost all infectivity, and most particles remaining after treatment appeared normal. Some of the rings from alkali and from sodium dodecyl sulphate treatments appeared to have one or more angular corners, in contrast to the very well-defined circular rings in Pl. 1, fig. 1. Perhaps the true cross-section is in fact hexagonal, obscured in Pl. 1, fig. 1 by a coating of some substance which is removed by the alkali and sodium dodecyl sulphate treatments. Alternatively the angular outline might have been produced by the alkali and sodium dodecyl sulphate treatments removing material formerly an integral part of the particles, or the angular rings may have been formed by small degradation products re-aggregated in the manner suggested by Harrington & Schachman (1956) for tobacco mosaic virus. Rings in stained preparations appeared to be circular, even at very high magnifications (Pl. 4).

Particles were also degraded by treating them with aqueous phenol. Various concentrations were tried, but most information came from pictures of a virus suspension in 0·1 % (w/v) phenol allowed to react at room temperature for 24 hr. Plate 2, fig. 5, illustrates such a preparation and shows that the ends of many of the particles had disintegrated, revealing the tubular structure very well. It is difficult to measure the diameter of the central hole accurately, as the particles tend to collapse inwards, but estimates suggest that it is about

6 mμ in diameter. A few particles had thread-like material projecting from them; one is shown in the upper right corner of Pl. 2, fig. 5. This thread-like substance may well be the nucleic acid; treatment with more concentrated phenol has been used to isolate infective nucleic acid preparations from tobacco mosaic virus (Gierer & Schramm, 1956). The appearance of the phenol-treated virus varied characteristically, both between duplicate mounts and in different regions of the same mount. In some preparations all gradations from apparently undamaged particles to completely disrupted ones were found in a width of a few hundred microns on the mount. This variability may have been caused by local high concentrations of phenol on those parts of the grid to which the last traces of liquid receded as the drop dried; in these regions the attack would be more severe, for the water would evaporate preferentially. The particles might thus have been attacked as they lay on the mount, the fragments remaining nearby as the liquid dried.

Stained preparations

Stained preparations were usually examined as gross drop mounts on thin carbon films, and in the following descriptions this technique can be assumed to have been used unless another is specifically mentioned. The staining treatments caused considerable aggregation; osmium-stained preparations retained about half their infectivity, but the other reagents, probably because of their acidity, destroyed almost all of the infectivity. Stained preparations usually gave good contrast in the electron microscope, permitting accurate focusing at × 80,000, but it was sometimes necessary to work at 60 kV. and to use a high contrast developer to obtain images which could be printed satisfactorily. Plates 2–4 record typical results and show that two main types of image were produced. Osmium tetroxide, phosphotungstic acid and phosphomolybdic acid gave a densely stained central core 6–7 mμ wide, that is, about one-third of the width of the particle, whereas with uranyl acetate and with lanthanum nitrate the core was less dense and only 4–4·5 mμ wide. The outside edges and the boundaries of the central cores were clearly defined, allowing the total and core widths to be measured (Table 1). In unstained control preparations, the central core could only just be seen (Pl. 2, fig. 6), and this is probably the reason for the high error for the estimate of the core width in unstained particles. After treatment with neutralized phosphotungstate or phosphomolybdate followed by spraying on to mounts (Brenner & Horne, 1959), many particles showed visible changes in width along their lengths; no useful estimate of particle width was possible on these specimens. Differences in total width of the same order are also found with the various stains (Table 1).

Rings were common in the phosphotungstic acid-stained preparations, and the central dark area in them corresponded in size to that of the central band in similarly treated rods. It was also approximately equal to the size of the central hole in the rings from the virus preparations treated with alkali or sodium dodecyl sulphate. Sometimes phosphotungstic acid-stained particles showed a slightly different picture, with a lightly stained central region having narrow but densely stained edges (Pl. 4, fig. 13). We think that this indicates that the stain had been partially removed, for it can easily be washed out either by dialysis or by repeated sedimentation and resuspension in distilled water. Particles which have been stained with phosphotungstic acid and then washed free from stain in this way can be re-stained with phosphotungstic acid, but less intensely than before. They can also be re-stained with uranyl acetate, when they show the narrow central band characteristic of that stain, so that deposition and subsequent removal of phosphotungstic acid seems not to remove material other than the stain itself from the centres of the particles. Further evidence for this conclusion is provided by the ultraviolet absorption spectrum of such stained and washed particles which closely resembled that of untreated virus particles. Raising the concentration of phosphotungstic or phosphomolybdic acids or of uranyl acetate to 5% had little effect on the intensity of staining; with concentrations of stain less than 2% (w/v), the staining became progressively weaker, but even with 0·01% (w/v) phosphotungstic acid there was still some staining. The pH values given in Table 1 are those for aqueous solutions of the reagents at the concentrations stated. Phosphotungstic and phosphomolybdic acids stained less intensely at pH 6·5–7·0, but uranyl acetate stained little when the pH was raised to pH 5·5.

Table 1. *Estimates of total width and core width of tobacco rattle virus particles treated with various agents*

Treatment	Mean total width (mμ)	Mean core width (mμ)
Unstained	21·84 ± 0·31	4·18 ± 1·00
2% (w/v) Lanthanum nitrate; pH *c.* 4·0	22·65 ± 0·10	3·99 ± 0·10
1% (w/v) Osmium tetroxide; pH 5·5	22·36 ± 0·71	5·94 ± 0·055
2% (w/v) Phosphomolybdic acid; pH 2·0	17·16 ± 0·064	6·98 ± 0·036
2% (w/v) Phosphotungstic acid; pH 3·0	19·99 ± 0·22	5·96 ± 0·26
2% (w/v) Uranyl acetate; pH 4·0	19·94 ± 0·33	4·39 ± 0·25

Enzyme treatments. Incubation with trypsin under the conditions used for these experiments had little effect upon infectivity. Ribonuclease at 1 mg./l. strongly inhibited infection, and even at 0·1 mg./l. it decreased lesion numbers to about a quarter of the control values. However, ribonuclease did not destroy infectivity *in vitro*, because infectivity was restored by diluting the mixtures. Also, samples incubated for 4 hr. at 37° with ribonuclease (0·1 mg./l.) were as infective as samples to which the ribonuclease was added immediately before inoculation. Particles incubated with trypsin or ribonuclease and then stained with phosphotungstic acid were indistinguishable from similarly stained ones which had not been treated with the enzymes; incubation with ribonuclease had no effect upon the appearance of particles subsequently stained with uranyl acetate.

Transverse bands. Particles stained with phosphomolybdic acid at pH 3·0 appeared similar to those treated with phosphotungstic acid, except that they showed a regular array of transverse bands (Pl. 3, fig. 9). After this had been found, careful inspection revealed traces of a similar structure in some pictures of phosphotungstic acid-stained virus particles. Particles incubated with trypsin before staining with uranyl acetate also showed transverse bands (Pl. 3, fig. 11). These structures were observed on a number of occasions, and when seen they could usually be detected on all the particles in a field and always in several plates of a through-focus series. The dark bands cross the particles and were separated by lighter areas which are approximately equal to them in width. In some pictures there was a suggestion that the dark bands were not quite at right angles to the long axes of the particles, but it is difficult to be certain of this because often they could not be traced across the heavily stained centres of the particles. Estimates of the spacing of the dark lines, obtained by measuring tracings of high magnification high-contrast images, are given in Table 2. If the dark and light bands are assumed to be of equal width, each must be 1·2–1·3 mμ wide.

Table 2. *Spacing of the transverse bands on stained particles of tobacco rattle virus*

Stain	Spacing (mμ)	Number measured
Phosphomolybdic acid	2·59	120
Phosphotungstic acid	2·44	53
Uranyl acetate	2·50	192
Mean	2·52	365 (total)

DISCUSSION

The tubular nature of tobacco rattle virus particles is clearly evident from the shadow-cast pictures of partly-degraded material (Pls. 1, 2). These plates also show how closely the tobacco rattle virus particle resembles tobacco mosaic virus in its general appearance; we have already drawn attention to the resemblances in some other properties (Harrison & Nixon, 1959). The pictures suggest that the central hole may be about 4 mμ in diameter, as in shadow-cast preparations of purified virus (Pl. 1, fig. 1) or rather larger (about 6 mμ) in particles treated with alkali, sodium dodecyl sulphate or phenol. The projecting core of the particle in Pl. 1, fig. 3, is about 15 mμ in diameter; but whereas rings with 6 mμ holes are abundant, particles with 15 mμ cores are rare, and their structural significance, if any, is unknown. The diameter of the holes in rings, and the width of the central cores in stained particles, also fall into two groups. The simplest explanation, namely that particles stained with osmium tetroxide, phosphotungstic acid or phosphomolybdic acid all have material removed from their centres by the staining treatment, and that the resulting cavity is then filled loosely with stain, cannot be true; for although it is possible to remove phosphotungstic and phosphomolybdic acids by dialysis, such particles then show a narrow core when restained with uranyl acetate. Moreover, particles stained with phosphotungstic acid and then dialysed free from stain still have a similar ultraviolet absorption spectrum to that of untreated virus preparations. And osmium tetroxide stained particles, which appear stained in a manner similar to those treated with phosphotungstic and phosphomolybdic acids, still retain considerable infectivity. The substance lining the central hole thus differs from the rest of the particle in staining heavily with osmium tetroxide, phosphotungstic acid or phosphomolybdic acid and also in being readily removed by phenol and other agents.

The results from examining stained and partially-degraded particles fit together very well, and it is reasonable to suppose that both reflect the structure of the intact particle. The diagram (Fig. 1) summarizes these observations. The great similarity in appearance and many other properties between tobacco rattle virus and tobacco mosaic virus makes it seem reasonable to suppose that the two particles have a similar internal structure. The internal structure of tobacco mosaic virus has been intensively studied by X-ray diffraction, and the particle has been shown to consist of structurally equivalent protein subunits with a molecular weight of about 18,000 arranged in a helix about the long axis of the rod, the pitch of the helix being 2·3 mμ and the maximum diameter 18 mμ. The outside of the particle is grooved, so that the apparent width is decreased to 15 mμ when particles pack closely together. Estimates of radial density indicate that the phosphate-sugar 'backbone' of the ribonucleic acid lies 4·0 mμ distant from the centre of the rod (Holmes & Franklin, 1958). Assuming that tobacco rattle virus has a similar structure to tobacco mosaic virus, but with slightly different dimensions, the transverse bands we have observed on rods may well represent the protein helix of that particle. Early attempts to stain tobacco mosaic virus in a similar manner invariably failed to show more than the central core first observed by Huxley (1957), and even this is not always visible, suggesting that perhaps it may only be seen on partly-degraded particles. However, we have now been able to stain tobacco mosaic virus with phosphomolybdic acid, and such preparations give pictures essentially similar to those of tobacco rattle virus shown here, although

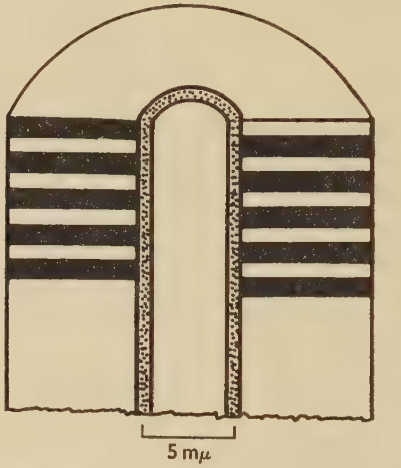

Fig. 1. Diagram to show the structure of the tobacco rattle virus particle as revealed by electron microscopy.

tobacco mosaic virus has less affinity for stain than tobacco rattle virus (Pl. 2, fig. 7). The work on stained tobacco mosaic virus will be described elsewhere.

Our experiments provide no direct evidence about the position of the nucleic acid within the tobacco rattle virus particles. However, they do suggest that the outer part of tobacco rattle virus must differ from the parts adjacent to the central hole. Further, the ready accessibility of these inner parts of the tobacco rattle virus particle to strains suggests that the nucleic acid is not here, for if it were it might be accessible to enzymic digestion, and the virus is resistant to both trypsin and ribonuclease *in vitro*. Another suggestion, which we consider to be more plausible, is that the nucleic acid is located in that part of the particle which we have been able to stain least, namely the region which lies immediately outside the part which lines the central hole. Perhaps this part of the particle has a low affinity for stain because it is the densest part, and it is interesting to observe that when the structure of tobacco mosaic virus is scaled up by the ratio of the total widths of tobacco rattle virus: tobacco mosaic virus, then the nucleic acid would occupy a position 5 mμ from the centre, which is, in fact, that part of the tobacco rattle virus particle which stains least.

We wish to acknowledge the assistance of Mr R. D. Woods.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Tobacco rattle virus particles from a purified preparation. Gross-drop mount on collodion, shadowed with platinum. Two short fragments of rod lie end-on and show the central hole, approximately 4 mμ in diameter. × 150,000.

Fig. 2. Particles from a preparation treated with 0·02 % (w/v) sodium dodecyl sulphate at pH 7·0 for 30 sec. at 60°. Gross-drop mount on collodion shadowed with platinum. Two fragments of rod lie end-on. They have an angular outline, with a central hole approximately 6 mμ in diameter. × 150,000.

Fig. 3. Particles from a preparation heated in 0·1 M-sodium borate + carbonate buffer (pH 10·3) for 10 min. at 45°. Gross-drop mount on collodion, shadowed with platinum. Towards the lower right one particle shows a projecting core 15 mμ in diameter. × 150,000.

Fig. 4. Particles from a preparation heated with 0·1 M-sodium borate + carbonate buffer (pH 10·3) for 5 min. at 46°. Gross-drop mount on collodion film, shadowed with platinum. Two short fragments lie end-on. They have an angular outline and a central hole approximately 6 mμ in diameter. × 150,000.

PLATE 2

Fig. 5. Particles from a tobacco rattle virus preparation treated for 24 hr. with 0·1 % (w/v) phenol at room temperature. Gross-drop mount on collodion film, shadowed with platinum. Many of the rods have damaged ends, showing the tubular structure. In the upper right corner, thread-like material, possibly nucleic acid, can be seen. × 150,000.

Fig. 6. Tobacco rattle virus particles from a purified preparation mounted on a carbon film. The central hole is just visible as a dark line on some particles. × 400,000.

Fig. 7. Particles from a mixed preparation of tobacco rattle virus and tobacco mosaic virus, stained with 2 % (w/v) phosphotungstic acid (pH 3·0). The relative widths of the two viruses and their different affinities for stain are clearly seen. × 400,000.

PLATE 3

Fig. 8. Tobacco rattle virus particles stained with 5 % lanthanum nitrate at pH 4·0. Carbon film, gross-drop mount. The particles are strongly outlined and the central hole is heavily stained. × 400,000.

Fig. 9. Tobacco rattle virus particles stained with 2 % (w/v) phosphomolybdic acid (pH 2·0). Gross-drop mount on carbon film. The particles have shrunk to a width of about 17 mμ, and both central hole and transverse banding are visible. × 400,000.

Fig. 10. Tobacco rattle virus particles stained with 2% (w/v) uranyl acetate (pH 4·0). Gross-drop mount on carbon film. The central hole and transverse banding are visible. × 400,000.

Fig. 11. A similar preparation to fig. 10, but stained after incubation with trypsin. The transverse bands are now more densely stained than the central hole. × 400,000.

PLATE 4

Fig. 12. Tobacco rattle virus particles stained with 2 % (w/v) phosphotungstic acid (pH 3·0). The wide central region is heavily stained, but no transverse banding is visible. × 400,000.

Fig. 13. Tobacco rattle virus particles, stained with 2 % (w/v) phosphotungstic acid (pH 6·0). Gross-drop mount on carbon film. The stain has been partially removed from the particles during washing, but the central region is still clearly outlined. × 400,000.

Fig. 14. Tobacco rattle virus particles, stained with 2 % (w/v) phosphotungstic acid (pH 7·0) and mounted on carbon film by spraying without washing. The particles show typical staining like those in Pl. 4, fig. 12, and several fragments are seen end-on. There is some indication that the outside surface is irregular. × 400,000.

Fig. 15. Tobacco rattle virus particles, stained with 1 % (w/v) osmium tetroxide at pH 5·5. The staining pattern resembles that shown in Pl. 4, fig. 12, and no transverse banding visible. Particles treated in this way retain considerable infectivity. × 400,000.

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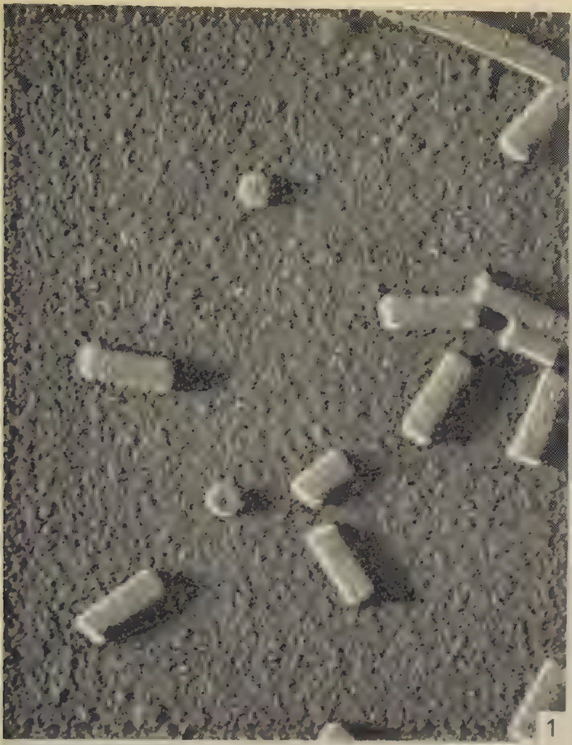

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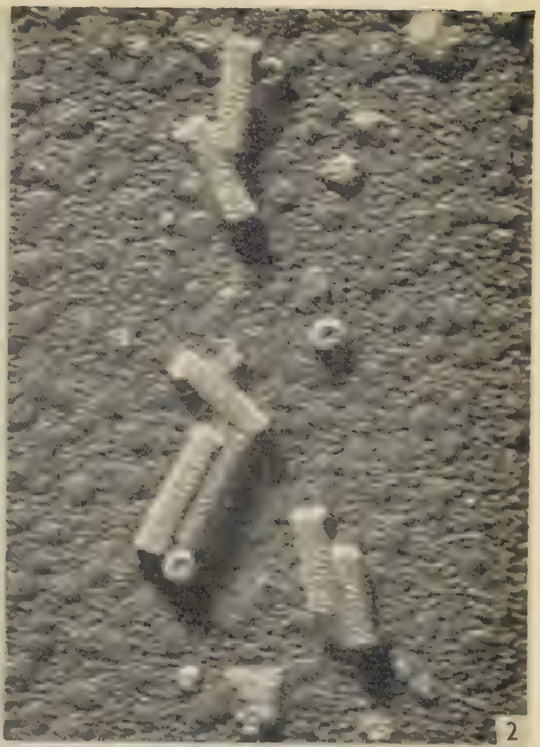

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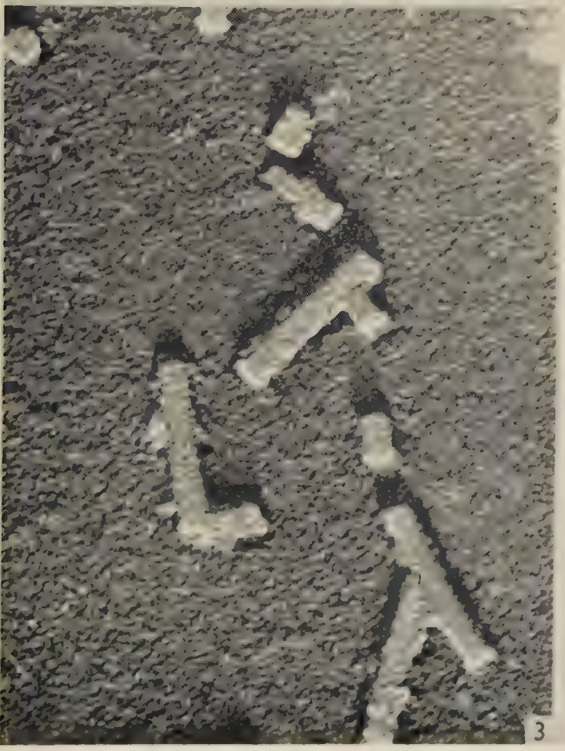

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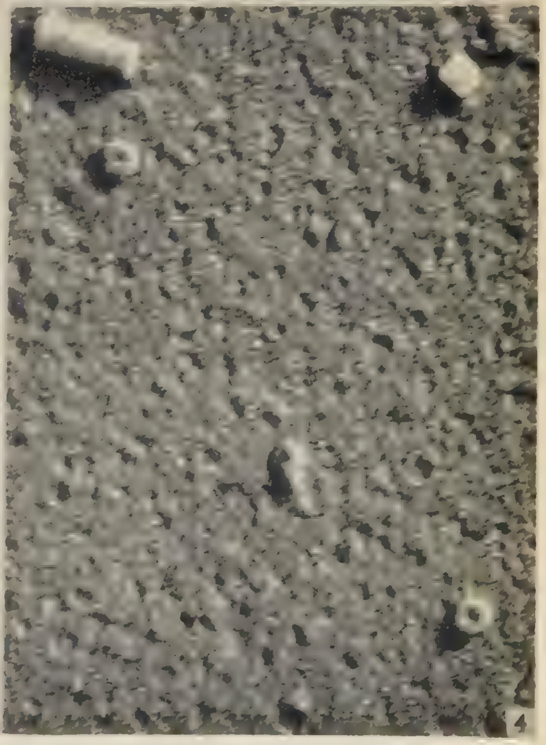

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H. L. NIXON & B. D. HARRISON—STRUCTURE OF TOBACCO RATTLE VIRUS. PLATE 1

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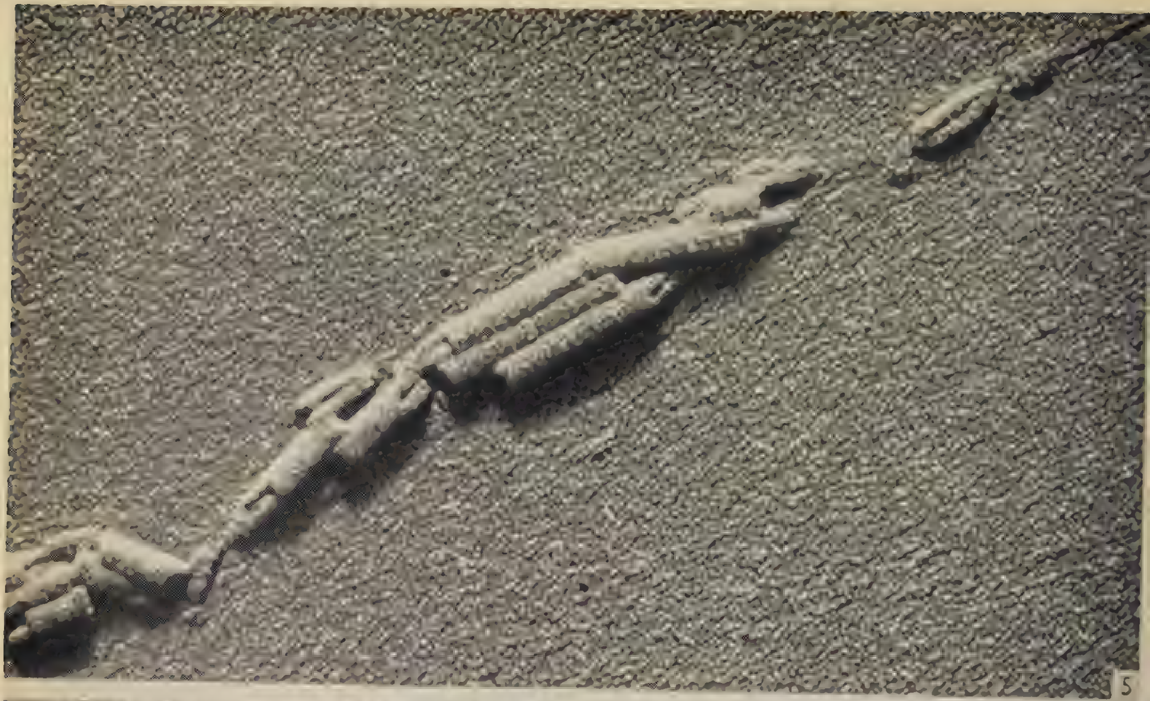

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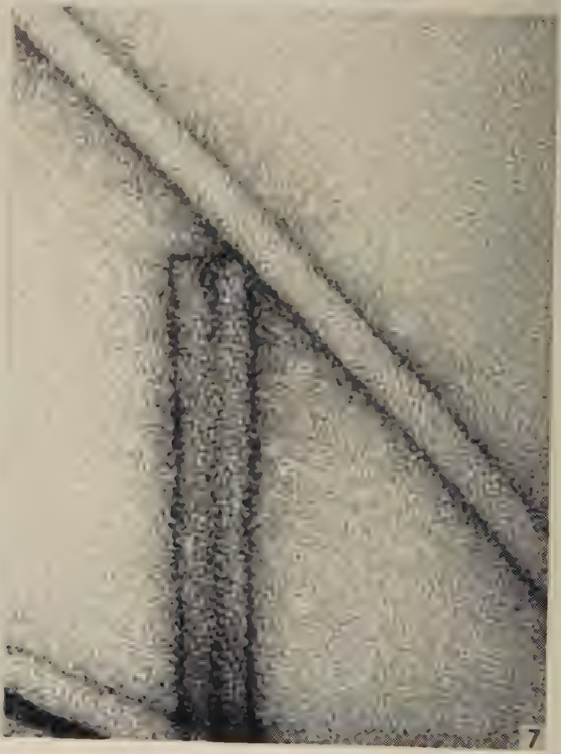

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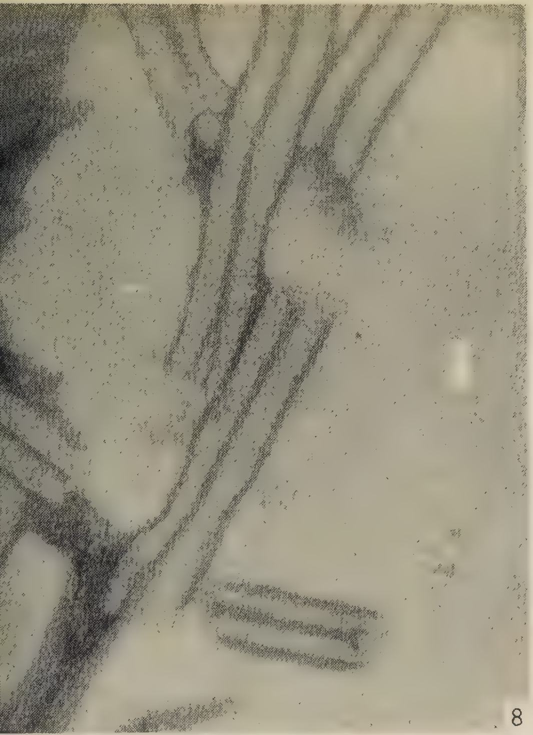
H. L. Nixon & B. D. Harrison—Structure of tobacco rattle virus. Plate 2.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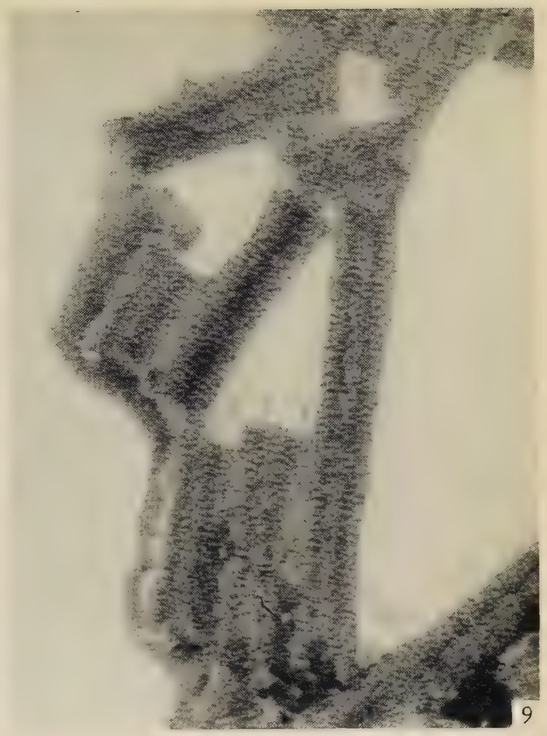
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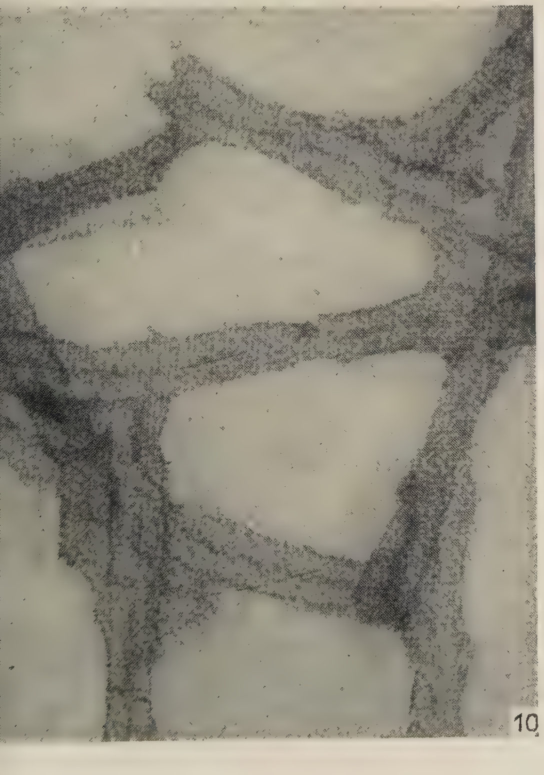
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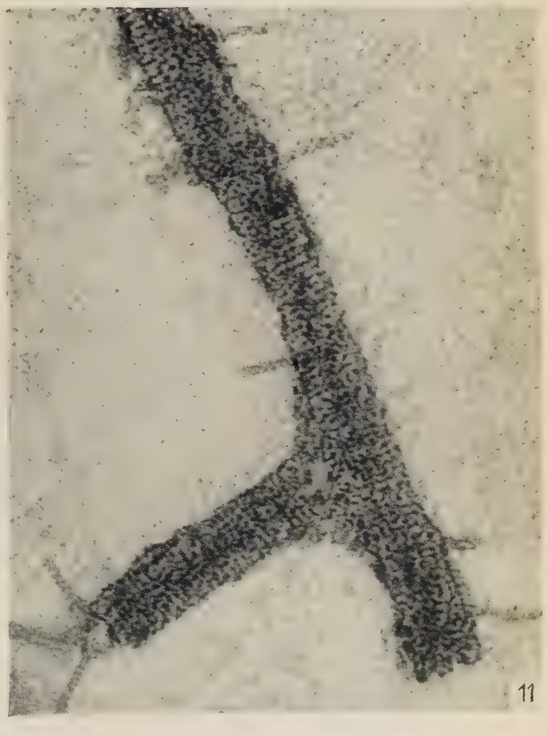
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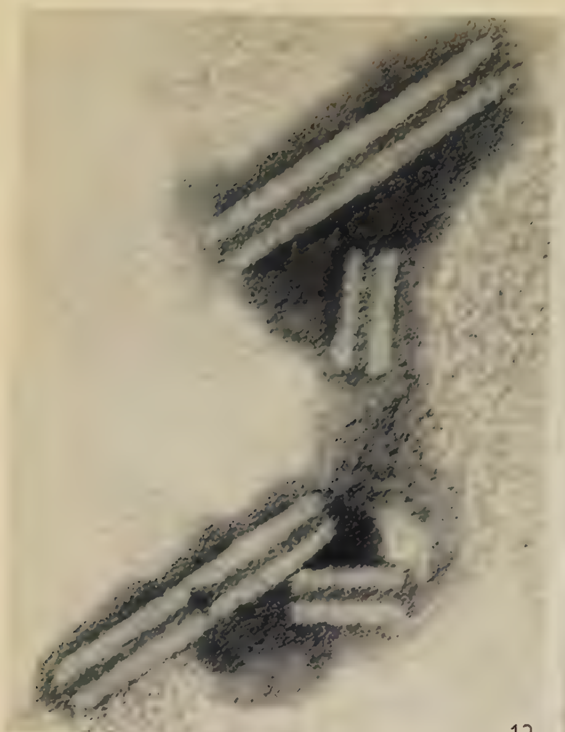
H. L. NIXON & B. D. HARRISON—STRUCTURE OF TOBACCO RATTLE VIRUS. PLATE 3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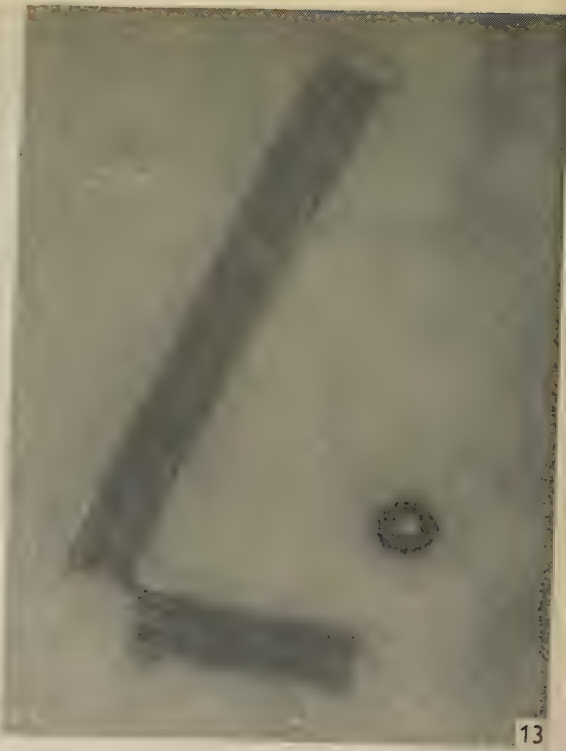
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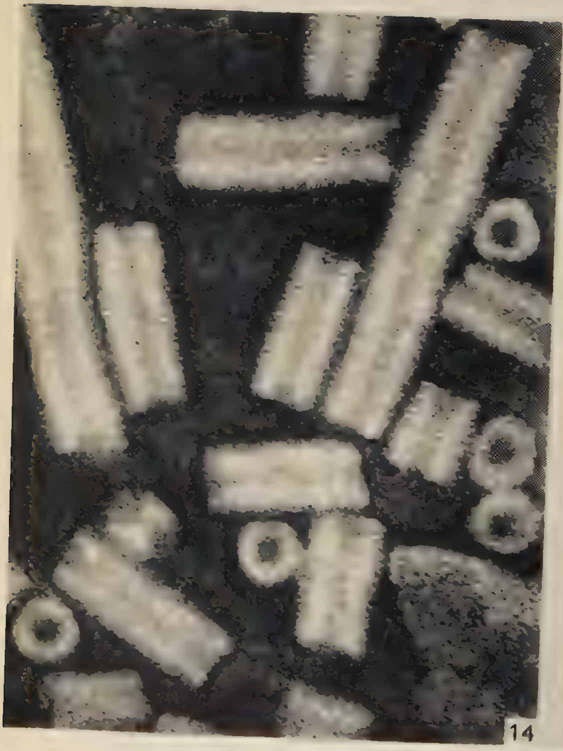
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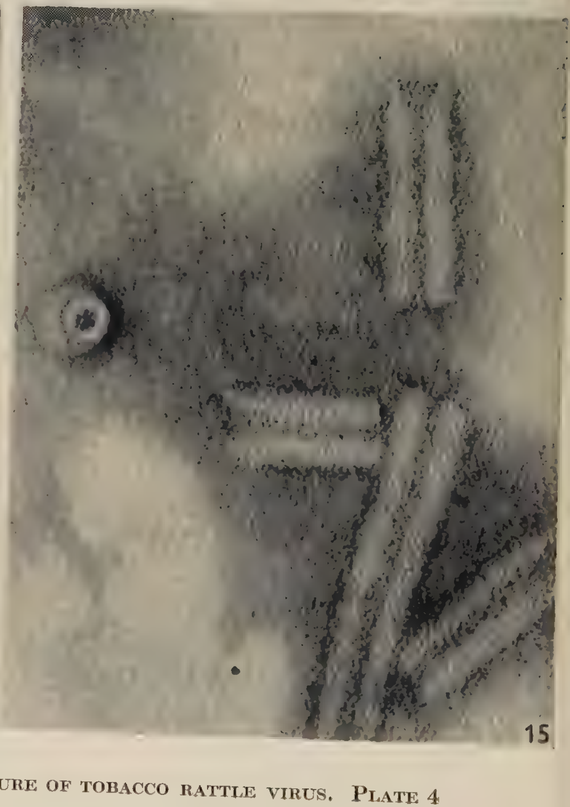
15

H. L. Nixon & B. D. Harrison—Structure of tobacco rattle virus. Plate 4

HARRISON, B. D. & NIXON, H. L. (1959). *J. gen. Microbiol.* **21**, 591–599

Some Properties of Infective Preparations made by Disrupting Tobacco Rattle Virus with Phenol

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SUMMARY: Preparations made by treating tobacco rattle virus with phenol were about 5% as infective as the initial virus suspensions when assayed on French bean, but less on tobacco. Virus nucleic acid seems to be a major constituent of such preparations. By constrast with whole virus, nucleic acid preparations lost infectivity when incubated for 20 min. with 0·02 mg. ribonuclease/l. or when stored for a day at 20°. Nucleic acid preparations contain threads about 1 mμ in diameter but very few or no virus rods. The infectivity of nucleic acid preparations was little affected by high-speed centrifugation for periods in which more than 95% of whole virus was sedimented. Ultraviolet-irradiated nucleic acid preparations were photoreactivable although irradiated preparations of whole virus were not. Nucleic acid preparations were only infective when made from purified virus suspensions or from frozen and clarified saps when these contained infective rod-shaped particles 179–192 mμ long. Phenol treatment of purified non-infective particles 73–77 mμ long yielded non-infective preparations.

Since the work on tobacco mosaic virus by Fraenkel-Conrat (1956) and by Gierer & Schramm (1956), infective nucleic acid preparations have been obtained from several other plant and animal viruses. Among plant viruses, these preparations have been made from purified samples of tobacco ringspot (Kaper & Steere, 1959) and potato *X* (Bawden & Kleczkowski, 1959*a*) viruses which have particles of quite different shape and stability from those of tobacco mosaic virus. A feature common to these nucleic acid preparations, whether they are made by treatment with phenol (Gierer & Schramm, 1956), heating with detergent (Fraenkel-Conrat, Singer & Williams, 1957) or heating in buffer (Kaper & Steere, 1959), is that their infectivity is usually less than 1% of that of the initial virus suspensions and is destroyed by minute amounts of ribonuclease. The present paper describes some properties of nucleic acid preparations made by the phenol method from a second rod-shaped plant virus, tobacco rattle. These preparations seem considerably more infective, relative to whole virus, than those obtained from other viruses.

METHODS

Tobacco rattle virus was 'purified' as described previously (Harrison & Nixon, 1959). Phenol treatment (Gierer & Schramm, 1956) was as follows: 5 ml. tobacco rattle virus suspension (200 mg./l.) in M/60 (pH 7·0) phosphate buffer was vigorously shaken for 2 min. with an equal volume of water-saturated phenol and the mixture then centrifuged for 3 min. at 9000 *g*. The aqueous top layer was removed from the centrifuge tubes to within 5 mm. of the interface with the bottom layer and shaken with 50 ml. ether in a separating funnel. The bottom layer of liquid was drawn off, and, in most experiments, the ether treatment repeated. The final preparations are referred to below as 'nucleic acid preparations'. The centrifuge and all solutions were kept at 5° before use. Infectivity was assayed on French bean (*Phaseolus vulgaris* L. var. Prince) or tobacco (*Nicotiana tabacum* L. var. White Burley) (Cadman & Harrison, 1959). 'Super-floss Celite' powder (Johns-Manville Ltd.) was mixed with all inocula.

RESULTS

Stability of infective preparations

Table 1 shows that after allowing for the differing susceptibility of different batches of test plants, the infectivity of different nucleic acid preparations relative to virus was very similar. Although infectivity decreased little in 20 min. at room temperature, only small and variable amounts remained after 20 hr. The variability may possibly be due to the presence of minute but differing amounts of stabilizing or inactivating substances in different preparations; such substances perhaps come from the glassware. At least 10% of the initial infectivity remained after nucleic acid preparations were frozen for 20 hr., and up to half remained after dialysis for 20 hr. at 5° against distilled water or 0·1 M-ammonium acetate. By contrast with nucleic acid preparations, untreated tobacco rattle virus lost little or no infectivity when kept for 20 hr. at room temperature; when mixed with nucleic acid prepara-

Table 1. *Effect of ageing and of ribonuclease on infectivity of nucleic acid preparations or purified virus*

The nucleic acid preparations were made from suspensions containing approximately 200 mg. tobacco rattle virus/l. Thus the virus suspension at 1/500 was at a concentration of 0·4 mg./l.

		Storage tem-perature	Infectivity*		
Inoculum	Dilution		Expt. 1	Expt. 2	Expt. 3
		(*a*) Inoculated after 20 min.			
Nucleic acid	1/2	5°	10,600	10,200	11,400
Nucleic acid	1/20	5°	913	463	—
Nucleic acid	1/2	20°	7,330	11,250	—
Nucleic acid + 0·02 mg./l. ribonuclease	1/2	20°	48	0	6
Virus	1/500	20°	1,000	1,000	1,000
		(*b*) Inoculated after 20 hr.			
Nucleic acid	1/2	−10°	—	1,840	1,000
Nucleic acid	1/2	20°	30	3	248
Virus	1/500	20°	1,000	1,000	1,000
Virus	1/2500	20°	—	121	256
Virus + 0·02 mg./l. ribonuclease	1/500	20°	425	486	1,320
Virus + nucleic acid (at a dilution of 1/2)	1/500	20°	—	122	376

* Infectivity was assayed on eight half-leaves of French bean. The total numbers of lesions counted were multiplied by the factor needed to bring the number for virus diluted 1/500 to 1000.

tions, however, the virus was less stable than in water or phosphate buffer and only about 10 % of its infectivity seemed to remain after 20 hr. at room temperature in this medium. Nevertheless, the stability of whole tobacco rattle virus, measured in this way, was considerably greater than that of the nucleic acid preparations.

Preparations of nucleic acid from tobacco mosaic virus vary in infectivity with dilution in quite a different way from untreated tobacco mosaic virus (Bawden & Pirie, 1957), but with tobacco rattle virus there is little difference in this respect between nucleic acid preparations and virus (Table 2). Reasonably accurate measurements of the infectivity of nucleic acid preparations from tobacco rattle virus relative to that of the virus suspensions from which they came can thus be made. Different preparations have given figures of 2–10 % when assayed on French bean, values of 4–6 % being commonest. The relative infectivity of the nucleic acid preparations, however, depends on the species of plant used for the assays, and values obtained with tobacco are less than those with French bean. For example, nucleic acid and virus inocula that gave 855 and 219 lesions, respectively, on French bean, gave 42 and 69 lesions on tobacco. Thus although French bean was more susceptible to the virus than tobacco, the difference was still greater for the nucleic acid preparations. Unusually great susceptibility to nucleic acid inocula may be a general property of French bean, for Commoner (1957) found that preparations of nucleic acid from tobacco mosaic virus which had a relative infectivity of 0·1–0·5 % when assayed on *Nicotiana glutinosa*, had values of 10 % or more on French bean. Tobacco mosaic virus-nucleic acid produced more lesions on French bean than on *N. glutinosa*.

Table 2. *Effect of dilution on infectivity of virus and nucleic acid preparations*

The nucleic acid preparations were made from suspensions containing 200 mg. virus/l. Thus the virus suspension at 1/100 contained 2 mg. virus/l.

		Infectivity*	
Inoculum	Dilution	Expt. 1	Expt. 2
Nucleic acid	1/2	3040	1580
Nucleic acid	1/10	973	455
Nucleic acid	1/50	167	80
Virus	1/100	2005	694
Virus	1/500	379	132
Virus	1/2500	79	43
% infectivity† $\frac{\text{Nucleic acid}}{\text{Virus}}$		4·6	6·5

* Total lesions produced on eight half-leaves of French bean.

† Calculated from the dilution required to produce 30 lesions/half leaf.

Effect of ribonuclease

Crystallized pancreatic ribonuclease (Armour Pharmaceutical Co.) at 0·02 mg./l. almost completely abolished the infectivity of nucleic acid preparations in 20 min. at room temperature, whereas at this concentration the enzyme had little effect on the infectivity of virus after 20 hr. (Table 1). The effect of ribonuclease on infectivity of virus inocula does not increase with time of incubation with the enzyme (Nixon & Harrison, 1959). Crystallized trypsin at 1 mg./l. was less effective in abolishing the infectivity of nucleic acid preparations than ribonuclease at 0·02 mg./l., and its action probably came from traces of ribonuclease still present. Chymotrypsin at 1 mg./l. (provided by Dr A. Kleczkowski), which was free from material able to hydrolyse yeast nucleic acid, had no measurable effect on the infectivity of nucleic acid preparations in 20 min. at room temperature.

Infective nucleic acid preparations can be made direct from tobacco sap which has been frozen and then clarified by low-speed centrifugation (Table 3). Their infectivity is destroyed by 20 min. incubation with ribonuclease (0·02 mg./l.) or by 20 hr. storage at 20°. The relative infectivity of preparations made from sap is, however, much less than those made from purified tobacco rattle virus and is usually about 0·2 % of that of the original sap.

Table 3. *Effect of aging and of ribonuclease on infectivity of sap or of a nucleic acid preparation made from sap*

Infective tobacco sap was stored for 2 days at −10°, then thawed and clarified immediately before use. The nucleic acid preparation from sap was made by shaking with an equal volume of water-saturated phenol; the subsequent procedures were as already described.

			Infectivity[*]	
Inoculum	Dilution	Storage temperature	inoculated after 20 min.	inoculated after 20 hr.
Nucleic acid	1/1	5°	136	1[†]
Nucleic acid + ribonuclease (0·02 mg./l.)	1/1·2	20°	1	—
Sap	1/1000	20°	64	67
Sap + ribonuclease (0·02 mg./l.)	1/1000	20°	50	67
Sap	1/5000	20°	—	24

[*] Total lesions produced on eight half-leaves of French bean.

[†] Kept at 20°.

High-speed centrifugation

Less than a quarter of the infectivity of nucleic acid preparations was sedimented by high-speed centrifugation in conditions in which over 95 % of the virus was sedimented (Table 4). The material sedimented from nucleic acid preparations seemed not to contain contaminating virus particles, for its infectivity was abolished by ribonuclease (0·02 mg./l.).

Electron microscopy

Nucleic acid preparations sometimes contained a very few virus rods, but comparison with virus preparations of known concentration showed that the rods were too few to account for even 1 % of the observed infectivity. Preparations dialysed for 20 hr. at 5° against distilled water or 0·1 M-ammonium acetate (pH 6·8) were mounted for electron microscopy by spraying on to freshly cleaved muscovite mica and shadowing with platinum. A layer of carbon was then deposited at normal incidence, followed by direct stripping on to a water surface and mounting on 4- or 7-hole platinum disks. The method is essentially that of Hall (1956), but the collodion and silicon monoxide films are replaced by a single layer of evaporated carbon. Samples dialysed against ammonium acetate contained slender fibrils 4–6 mμ in diameter, but there was also evidence of considerable mounts of much smaller material, which could be detected only by the alignment of the granules of platinum. These very fine threads appeared to be about 1 mμ in diameter, and the larger fibrils may well be aggregates of the threads. Samples dialysed against distilled water showed the fine threads but not the fibrils, which were replaced by irregular blobs which might well be more compact aggregates of the threads. Nucleic acid preparations made from tobacco mosaic virus by the phenol method and dialysed against water or ammonium acetate had the same appearance as those from tobacco rattle virus. In forming more compact aggregates in water than in ammonium acetate, the nucleic acid preparations behave like the nucleic acid fibres projecting from the ends of partially degraded rods of tobacco mosaic virus. When mounted in acetate these fibres were up to 2750 mμ long but when mounted in water they were thicker and much shorter (Hart, 1958). In one experiment a nucleic acid preparation from tobacco rattle virus was dialysed against an ammonium acetate/acetic acid mixture with the pH value (pH 4·8) of the distilled water used previously. This sample appeared very similar in the electron microscope to those suspended in pH 6·8 acetate, indicating that the samples in acetate or distilled water did not differ in appearance because of the difference in pH value. Hart (1958) suggested that the different appearances of nucleic acid fibres prepared in water or acetate may be caused by the effects of these media on the degree to which the fibres coil; this is also a plausible explanation of our observations.

Table 4. *Effect of high-speed centrifugation on infectivity of virus and nucleic acid preparations*

Three ml. nucleic acid preparation diluted 1/2, or virus preparation diluted 1/50, in M/60 phosphate buffer (pH 7) was centrifuged for 2 hr. at 5° and 47,000 *g* in a swing-out rotor. Samples were removed by carefully lowering to the required depth a Pasteur pipette whose tip was bent through an angle of 90°. Each sample was split into 2 parts; ribonuclease to final concentration 0·02 mg./l. was added to one part and distilled water to the other. Samples were inoculated approximately 20 min. after making the mixtures.

	Infectivity*			
	Nucleic acid		Virus	
Position of sample in centrifuge tube	Without ribonuclease	With ribonuclease	Without ribonuclease	With ribonuclease
Top 1·2 ml.	1365	1	7	9
Bottom 1·0 ml.	1105	1	35	19
Dregs and re-suspended pellet	877	0	1630	844

* Total lesions produced on 8 half-leaves of French bean.

Photoreactivation of ultraviolet-irradiated preparations

Tobacco mosaic and tobacco rattle are the only two plant viruses tested whose residual infectivity after ultraviolet-irradiation is the same whether assayed on plants kept in daylight or in darkness after inoculation; that is, these viruses are not photoreactivable (Bawden & Kleczkowski, 1955; Cadman & Harrison, 1959). Recently, however, Bawden & Kleczkowski (1959*b*) showed that ultraviolet-irradiated *nucleic acid preparations* from tobacco mosaic virus are photoreactivable; Table 5 provides evidence that nucleic acid preparations from tobacco rattle virus are also photoreactivable. The inactivation curve obtained by plotting residual infectivity against dose of ultraviolet radiation was exponential whether or not the assay plants were kept in daylight after inoculation, but when they were kept in darkness its slope was steeper. The ratio of the slopes of the two inactivation lines varied from 1·4 to 1·9 in different experiments, values comparable with those obtained with other viruses and with nucleic acid preparations from tobacco mosaic virus (Bawden & Kleczkowski, 1955, 1959*b*).

Table 5. *Effect of ultraviolet irradiation on the infectivity of a nucleic acid preparation*

Two ml. nucleic acid preparation were exposed to ultraviolet radiation for various times at 1450 μW/cm.^{2} in a Petri dish of 5 cm. diameter.

'Dark' and 'light' indicate that the French bean test plants were kept after inoculation in darkness and daylight respectively.

Dose of ultraviolet radiation (sec.)	Dilution of nucleic acid preparation	Total number of lesions on eight half-leaves	
		Light	Dark
0	1/20	69	42
0	1/80	12	10
20	1/2	77	21
30	1/2	30	6

Preparations from short virus particles

Harrison & Nixon (1959) found that the lengths of particles of tobacco rattle virus fell predominantly into two categories, 73–77 and 179–192 mμ, and showed that only the long ones were infective. Both types of particle appeared to have the same gross chemical composition, and no differences were found between their proteins by serological methods. The phenol method offers a way of investigating the possibility that the essential difference between them lies in their nucleic acid components. Preparations of short particles were obtained from purified virus suspensions by rate zonal centrifugation in a sucrose density-gradient. When these were treated with phenol, the resulting preparations had only a trace of infectivity, corresponding exactly to the nucleic acid contributed by the few long particles contaminating the starting material. Evidently the short particles contain non-infective nucleic acid. Preparations made by phenol treatment of short particles appeared similar in the electron microscope to nucleic acid preparations from unfractionated samples of tobacco rattle virus.

DISCUSSION

By analogy with tobacco mosaic virus, nucleic acid can be expected to be a major constituent of the preparations obtained by treating tobacco rattle virus with phenol. Ultraviolet absorption measurements on the preparations are also consistent with this idea, and the sensitivity of infectivity to inactivation by ribonuclease suggests that the nucleic acid is of the ribose type, like that in all other plant viruses yet examined. Phosphorus analyses too, made by Dr W. S. Pierpoint, suggest that the nucleic acid preparations do in fact contain all, or nearly all, of the virus nucleic acid. Electron microscopy shows that the preparations contain little or no protein in the form of virus rods, and although small fragments of virus protein may well be present, ultraviolet absorption measurements suggest that the amount of protein contamination is small. The infective particles in nucleic acid preparations behave much like those from other viruses. They are inactivated by minute amounts of ribonuclease or by storage *in vitro* at 20°, and few of them are sedimented by high-speed centrifugation for periods in which over 95 % of normal tobacco rattle virus particles sediment. The infective particles may well be delicate thread-like structures of the type seen in the electron micrographs. Like those from tobacco mosaic virus, ultraviolet-irradiated nucleic acid preparations from tobacco rattle virus show the phenomenon of photoreactivation, although neither of the parent viruses does. Presumably the protein and nucleic acid are combined similarly in the two viruses, in a manner that prevents reversible changes occurring in their nucleic acids when the viruses are exposed to ultraviolet radiation. This type of combination may be common to viruses with straight rod-shaped particles.

Although infective nucleic acid preparations can readily be made from a wide range of ribose nucleic acid-containing viruses, such preparations usually appear much less infective than their parent virus suspensions. One possible reason is that an infective nucleic acid particle has a smaller chance than an infective nucleoprotein particle of multiplying when inoculated to a susceptible host. Indirect evidence of this is given by tobacco mosaic virus, nucleic acid preparations from which may increase thirty-fold in infectivity when mixed with virus protein in conditions favouring the formation of rod-shaped particles (Fraenkel-Conrat, 1957). A second reason is that the methods used for extracting the nucleic acid from virus particles may give small yields of high molecular weight material, as for example with potato virus *X* (Bawden & Kleczkowski, 1959*a*), or may result in damage to some initially infective nucleic acid particles. Furthermore, methods of making infective nucleic acid preparations take no account of the possibility that something in addition to nucleic acid may be needed for infectivity. There are, therefore, many possible explanations for the observation that the fraction of the infectivity of whole virus which remains in nucleic acid preparations is greater with tobacco rattle virus than other viruses. It has already been pointed out that French bean plants, which were used in work with tobacco rattle virus, may be unusually susceptible to infection by nucleic acid inocula. That virus protein can influence the chance of an infection succeeding is shown by experiments with two strains of tobacco mosaic virus, which caused different numbers of lesions per unit weight when compared as nucleoproteins, but similar numbers when compared as separated nucleic acids (Fraenkel-Conrat & Singer, 1957). It would be interesting to know whether infective units of nucleic acid from one virus have the same chance of multiplying as those from other unrelated viruses when tested in the same host species and conditions. Apparent differences in numbers of nucleoprotein particles inoculated for each infection obtained when different viruses are tested in this way may come from differences in the behaviour of their protein portions during the processes of infection. Nucleoprotein particles of tobacco rattle virus have an unusually low specific infectivity (Harrison & Nixon, 1959), but nucleic acid units derived from them appear to have a similar infectivity to those from other plant viruses.

Besides those mentioned in the text, we are indebted to Mr R. D. Woods for technical assistance with the electron microscopy.

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On the Nature of the Glutamic Dehydrogenase Produced by Inter-Allele Complementation at the *am* Locus of *Neurospora crassa*

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SUMMARY: A method for the 40-fold purification of glutamic dehydrogenase from *Neurospora crassa* is described. Strains which are homocaryotic for the alleles am^1, am^2 or am^3 produce no detectable glutamic dehydrogenase, but heterocaryons of composition am^1+am^2 or am^1+am^3 do possess the enzyme activity. Extracts of am^1+am^2 mycelia have 10%, or rather less, of typical wild-type activity when assayed at about 20°. Enzyme preparations from am^1+am^2 are distinguished from wild-type preparations in: (*a*) their capacity for thermal activation as the temperature is raised between 20° and 35°; (*b*) their low stability at 60°. Extracts of am^1+am^3 mycelia show 20–25% of typical wild-type activity. Enzyme preparations from am^1+am^3 are distinguished from wild-type in their lower affinity for glutamate, and they tend also to be more thermolabile than wild-type enzyme, though less so than am^1+am^2 preparations. Experiments on mixed enzymes showed no evidence for any effect of either kind of heterocaryon preparation on the properties of wild-type enzyme, or vice versa. It thus seems likely that the effects observed are due to differences in the enzyme molecules themselves. The significance of these observations for theories on the mechanism of inter-allele complementation is discussed.

When a series of different mutations all result in a deficiency in the same enzyme, and all fall within the same very short chromosome segment, they may reasonably be considered as belonging to the same genetic locus (Fincham, 1959). Most commonly, two such 'allelic' mutations will not complement each other to form a non-mutant phenotype when present together in the same cell. However, in a number of cases, both in *Neurospora crassa* (Pateman & Fincham, 1958; Woodward, Partridge & Giles, 1958), and in *Salmonella typhimurium* (Hartman, Hartman, Serman & Loper, 1958), where an extensive series of alleles at a locus has been examined, certain pairs of alleles have been shown to be capable of promoting enzyme formation jointly, though unable to do so individually. In previous papers (Fincham, 1959; Pateman & Fincham, 1958) it was reported that of eleven mutants at the *am* locus of Neurospora, all of which lack glutamic dehydrogenase activity, only two pairwise combinations of alleles (am^1+am^2 and am^1+am^3) produced the enzyme activity in heterocaryons. An important question in relation to the mechanism of enzyme formation through inter-allele interaction (complementation) is whether the enzyme so produced is normal in its properties. In this paper, evidence will be presented showing that the two pairs of complementary alleles at the *am* locus produce varieties of glutamic dehydrogenase which are distinguishable from the wild-type enzyme, and from each other.

METHODS

Neurospora strains. The wild-type strain ST *A* of *Neurospora crassa* was the same as used in previous studies (Fincham & Pateman, 1957*a*), and the wild-types 2*a* and 5*a* were closely related to it through inbreeding, as were most of the other strains used in this work. Double mutants carrying both an *am* allele and either *arg*-1 or *arg*-10 were the same as used in a previous study (Pateman & Fincham, 1958), with the exception of am^3 *arg*-1–2, which was made by the same procedure as the other double mutants. The use of the mutations at the two *arg* loci, which correspond to two different steps in arginine synthesis (Newmeyer, 1957), permitted the forcing of heterocaryons. Since *am* strains grow, after a lag, on minimal medium, it is not always possible to be certain that one has a heterocaryon when *am* single mutants are used. The four heterocaryons used in the present experiments were as follows: am^1 *arg*-10–13 + am^3 *arg*-1–2 (*het A*), am^1 *arg*-1–1526–78 + am^3 *arg*-10–14 (*het* B), am^1 *arg*-10–13 + am^2 *arg*-1–1509–78 (*het* C), am^1 *arg*-1–1526–78 + am^2 *arg*-10–1505–56 (*het* D). The alleles am^1, am^2 and am^3 are those formerly referred to as am^{32}, am^{47} and am^{29} respectively (Pateman & Fincham, 1958).

Enzyme assays. The standard assay system consisted of : 0·005–0·2 ml. of enzyme preparation diluted to 2·65 ml. with 0·1 M-sodium phosphate buffer (pH 8·0), 0·1 ml. 0·2 M-disodium α-ketoglutarate; 0·2 ml. reduced triphosphopyridine nucleotide (TPNH) to give an optical density of 0·35–0·40 at 340 mμ in the final mixture; 0·05 ml. 0·4 M-ammonium chloride; the mixture was held in a 1 cm. Unicam SP 500 spectrophotometer cell. The optical density was read at 340 mμ before addition of the NH_4Cl, which was added at zero time on a Perspex stirrer. Readings were taken at 15 sec. intervals for 3–5 min., and the initial rate of decrease of optical density was estimated graphically. A change in optical density of 0·001 in 1 min. was taken as the unit of activity, and specific activity was expressed as units/mg. protein. In the assay of crude extracts, and ammonium sulphate fractions which had not been dialysed, the TPNH was added last. Except in the experiments illustrated by Tables 1–3, where assays were carried out at room temperature (about 20°), temperature was controlled by a Unicam constant temperature housing attached to the spectrophotometer. While the standard assay made use of the back reaction (α-ketoglutarate → glutamate), the rate of the forward reaction was measured in a number of experiments. Here the following standard system was used: enzyme diluted to 2·6 ml. in 0·1 M-sodium phosphate buffer (pH 8·0); 0·2 ml. 0·5 M-monosodium glutamate; 0·2 ml. 0·16 % (w/v) triphosphopyridine nucleotide (TPN). The TPN was added at zero time and the rate of increase in optical density measured. With the standard reaction mixtures the back reaction was about six times as fast as the forward reaction for the same amount of enzyme.

Enzyme preparations. Cultures were grown from heavy inocula of conidia for 48 hr. at 25° in 100 ml. lots of liquid medium N (Vogel & Bonner, 1956) in Roux bottles. The resulting mycelium was washed with water, blotted, roughly weighed (the usual yield was 1–1·5 g./bottle) and ground in a chilled mortar with powdered glass and about 10 vol. of 0·05 M-sodium phosphate buffer (pH 8·0). The homogenate was filtered through a pad of kieselguhr by suction. Where further purification was intended, about 1 g. kieselguhr was used for each g. damp weight mycelium. The use of thicker pads of kieselguhr led to some loss of enzyme, but also to a higher specific activity in the filtrate (cf. the values for crude filtrates of wild type in Tables 1 and 2; *c.* 2–3 g. kieselguhr was used/g. mycelium in the experiments recorded in Table 2). Except where otherwise stated, the data of the present paper refer to enzyme preparations partially purified by the following method. To each 100 ml. of crude filtrate, 89 ml. saturated ammonium sulphate solution (70 g. dissolved in 100 ml. water) was added, and the precipitate was removed by centrifuging. To the supernatant fluid 13·3 g. solid ammonium sulphate was added; the precipitate was collected by centrifuging and dissolved in 20–30 ml. 0·05 M-sodium acetate buffer (pH 5·6). Each ammonium sulphate addition was made at close to 0°, over a 10 min. period, and with constant stirring. Centrifuging was for 10 min. at 3000*g* in each case. In some experiments (Table 2) the second ammonium sulphate precipitate was taken up in 0·05 M-sodium phosphate buffer (pH 8·0) and used as enzyme, with or without prior dialysis against the same buffer. To the fraction dissolved in acetate buffer, enough calcium phosphate gel was added to give 1–1·5 mg. calcium phosphate/mg. protein. The gel was prepared according to the method of Keilin & Hartree (quoted in Colowick & Kaplan, 1955), and aged for several months before use. After stirring at room temperature for 10 min., the gel was centrifuged down and resuspended in 10 ml. 0·05 M-sodium phosphate buffer (pH 6·5). After a further 10 min. stirring, the gel was again centrifuged and the supernatant fluid (first gel eluate) was saved. The elution was repeated as many times as necessary. Usually the second gel eluate had the highest specific activity, but sometimes the first or the third eluates were better. This procedure (Table 1) gave, in the case of wild-type enzyme, *c.* 40-fold increase in specific activity over the crude filtrate. For the am^1+am^2 and am^1+am^3 heterocaryons, the purification factors were usually only about 20-fold and 30-fold respectively, perhaps because of the lower stability of the enzyme in these mycelia. In some preparations of wild-type enzyme the ammonium sulphate fraction in acetate buffer was heated to 50° for 4 min., and the precipitate formed was removed

Table 1. *Purification of glutamic dehydrogenase from wild-type* Neurospora crassa

Fraction	Vol. (ml.)	Total protein (mg.)	Total units (thousands)	Specific activity
Filtered extract of 6 g. damp mycelium	50	325	110	340
Ammonium sulphate fraction	11	93	88	950
Gel supernatant fluid	11	—	31	—
First gel eluate	5	1·3	18	13,800
Second gel eluate	5	0·75	8	10,700

before addition of the gel. The inclusion of this step probably improved the procedure as a method for purifying wild-type enzyme (an over-all purification of 50-fold was obtained on one occasion), but it resulted in total loss of activity when used on am^1+am^2 preparations, so it was not generally used in the present work.

Protein determinations. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Table 2. *Thermostabilities of glutamic dehydrogenase preparations from* Neurospora crassa, *wild-type and heterocaryons*

		Protein	Specific activity		
Strain	Type of preparation*	content (mg./ml.)	Before heating	After heating†	Loss (%)
Wild type ST*A*	Crude filtrate	0·75	1070	1070	0
	Ammonium sulphate fraction	0·35	4540	2740	40
	Ammonium sulphate fraction, dialysed	0·35	3710	3140	15
am^1+am^2 *het* C	Crude filtrate	0·92	51	18	65
	Ammonium sulphate fraction	0·57	510	4	99
	Ammonium sulphate fraction, dialysed	0·56	261	42	85
am^1+am^2 *het* D	Crude filtrate	0·60	55	25	54
	Ammonium sulphate fraction	0·30	306	6	98
	Ammonium sulphate fraction, dialysed	0·33	103	30	71
am^1+am^3 *het* A	Crude filtrate	0·90	76	59	22
	Ammonium sulphate fraction	0·50	385	88	77
	Ammonium sulphate fraction, dialysed	0·51	300	183	40

* Crude extracts in 0·05 M-sodium phosphate buffer (pH 8·0); the same buffer was used for dissolving the ammonium sulphate fractions, and for dialysing.

† 15 min. at 60°.

RESULTS

Relative specific activities of wild type and heterocaryon preparations

Crude filtrates of wild-type organism have generally shown specific activities in the range 300–500, usually nearer the latter figure. Of the am^1+am^3 heterocaryons, *het* A and *het* B gave, in single experiments, specific activities in crude filtrates of 87 and 93 respectively. Corresponding values for the am^1+am^2 heterocaryons were 36 and 40 for *het* C, and 37 and 36 for *het* D. Where relatively thick pads of kieselguhr were used for filtration (as in the experiments recorded in Table 2) higher specific activities were generally obtained. The best estimates that can be given of average levels of enzyme activity are rather under 10 % for the am^1+am^2 heterocaryons, and 20–25 % of wild-type for the am^1+am^3 heterocaryons. Production of glutamic dehydrogenase seems to be unaffected by the *arg*-1 and *arg*-10 mutations in the heterocaryons; a heterocaryon formed between the two single mutants gave activity in the wild type range.

The differences in activity between wild-type and heterocaryon preparations were even more marked after partial purification, because of lower recoveries in the gel elution step in heterocaryon preparations. Recoveries of activity after ammonium sulphate fractionation were always high, and in some experiments on am^1+am^2 heterocaryons (e.g. *het* C in Table 2) significantly more than 100 % recovery of activity was obtained from the ammonium sulphate fraction. The possibility that, in these cases, new active enzyme was, in effect, being made by co-precipitation of materials contributed by the two alleles, was apparently ruled out by an experiment in which extracts of am^1 and am^2 mycelia grown separately and fractionated together yielded no activity. It seems probable that some activation of the enzyme from am^1+am^2 heterocaryons may occur during fractionation.

Table 3. *Thermostabilities of* Neurospora crassa *wild-type and heterocaryon* ($\mathrm{am}^1+\mathrm{am}^2$) *glutamic dehydrogenase preparations separately and in mixture*

Dialysed ammonium sulphate fractions in 0·05 M-sodium phosphate buffer (pH 8·0). Protein concentrations: 0·075 mg./ml. of wild-type (ST*A*) and 1·4 mg./ml. of *het* C protein.

Enzyme preparation	Time of heating at 60° (min.)		
	0	7	17
	Activity (units/0·2 ml.)		
Wild-type	44	42	34
Heterocaryon	30	6	2*
Mixture	70	50	32

* This rate probably corresponds to nearly zero activity, since no correction was made for unspecific oxidation of TPNH.

Heat stabilities

Enzyme preparations from both am^1+am^2 and am^1+am^3 heterocaryons were less stable to heat than those from wild-type organism. The thermolability of am^1+am^2 preparations was marked at all stages of purification; that of am^1+am^3 preparations was much less so. Rather consistent results have been obtained with crude filtrates and ammonium sulphate fractions (Table 2). Ammonium sulphate fractions of both wild-type and heterocaryons were much less stable than crude extracts, but were stabilized somewhat by dialysis. An experiment in which dialysed ammonium sulphate fractions of wild type and *het* C (am^1+am^2) were heated separately and in mixture showed a large difference in thermostability, without any significant effect of the one preparation on the stability of the other (Table 3). This indicates strongly that the difference in thermostability lies in the enzyme itself (or in something bound to the enzyme), and does not depend on, for example, differences in protein or salt concentrations.

Comparative work on heat stabilities of more purified fractions has encountered difficulties. Different gel eluates from the same source, or supposedly similar fractions prepared in different experiments, have sometimes differed markedly in stability, and it seems probable that differences in respect of traces of stabilizing or destabilizing substances, as well as in protein concentration, are involved. Different gel eluates may be more heterogeneous in these respects than cruder fractions. In spite of this variability, purified fractions from *am*^{1}+*am*^{2} heterocaryons have always been less stable to heat than comparable preparations from wild type.

Effect of temperature on reaction rate

A further peculiarity of enzyme from *am*^{1}+*am*^{2} was shown in experiments on the effect of temperature on the rates of the forward and back reactions. Some of the data obtained are shown in Fig. 1. Arrhenius plots for wild-type enzyme were not strictly linear, either for the forward or the back reactions, but tended to be slightly concave downwards, with a pronounced dip at the lower end of the temperature range. Such deviations from linearity are not uncommon in enzyme-catalysed reactions (Dixon & Webb, 1958). Over the range 25°–40°, where the curves are approximately linear, the apparent activation energies for the forward and back reactions catalysed by wild-type enzyme were about 11,400 and 8000 cal./mole respectively. The data for *am*^{1}+*am*^{3} are not significantly different from those for wild-type. In the case of *am*^{1}+*am*^{2} heterocaryon preparations, however, the apparent activation energies over the range 20°–30° were about twice as great as those for wild-type for both forward and back reactions. Up to about 30°, Q_{10} values of about 3, or even more, were characteristic of *am*^{1}+*am*^{2} enzyme, compared with less than 2 for wild-type enzyme. This was true in experiments both with

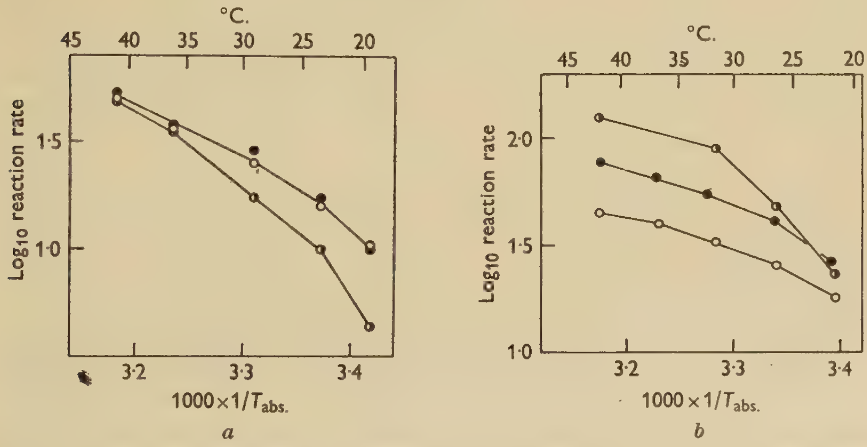

Fig. 1. Temperature dependence of forward and back reaction rates with various enzyme preparations. The amount of protein used/assay are given in parentheses. (*a*) Forward reaction: ●, wild-type 2*a* (4 μg.); ◑, *am*^{1}+*am*^{2} *het* C (38 μg.); ○, *am*^{1}+*am*^{3} *het* A (19 μg.). (*b*) Back reaction: ●, wild-type 2*a* (1 μg.); ◑, *am*^{1}+*am*^{2} *het* C (23 μg.); ○, *am*^{1}+*am*^{3} *het* B (7·5 μg.).

het C and with *het* D. Above 35° am^1+am^2 preparations became much more similar to wild-type in their response to temperature.

One possible explanation of this property of am^1+am^2 enzyme is that it tends to become converted to a more active form as the temperature is increased between 20° and 35°. This explanation is supported by experiments in which it was shown that the enzyme was more active after brief warming in the absence of substrates than when it had not been so treated, the temperature of assay being the same in both cases. Most of the extra activity was lost during 20 min. at 20°, but it could be regained by repeated warming (Fig. 2). The rate of the reaction catalysed by the activated enzyme tended to fall off rather rapidly (Fig. 2), as if activity were being lost during the course of the reaction. This capacity for reversible activation by mild heat is qualitatively similar to that shown by enzyme from am^{21} (Fincham, 1957), a mutant secondarily derived from am^2 (Fincham & Pateman, 1957*b*), though the effect is less in the present case. Wild-type enzyme shows no activation under the same conditions (Fincham, 1957).

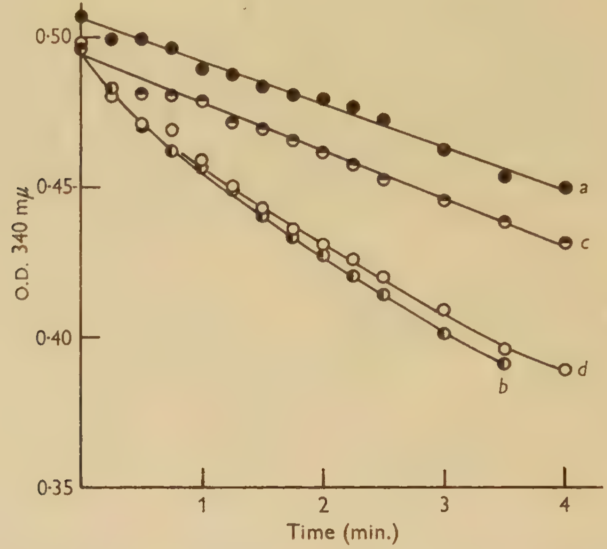

Fig. 2. Reversible activation of am^1+am^2 by warming. Activity assays at 20° on 80 μg. protein from *het* D: (*a*) no pretreatment; (*b*) enzyme in 0·1 M-sodium phosphate (pH 8), incubated 12 min. at 34°, cooled, and assayed within 2 min. (4 min. at 34° gave the same result); (*c*) enzyme 12 min. at 34° followed by 22 min. at 20·5°; (*d*) enzyme 12 min. at 34°, 22 min. at 20·5°, and a further 3 min. at 34°.

Experiments in which mixtures of wild-type and am^1+am^2 enzymes were assayed for activity at different temperatures gave no indication that the activity of either preparation was being affected by mixture with the other (Table 4). This, again, tends to support the hypothesis of a difference between two kinds of enzyme.

Table 4. *Effect of temperature on reaction rate with* Neurospora crassa *wild-type and* het *C* (am^1 + am^2) *glutamic dehydrogenase preparations separately and in mixture*

Each reaction mixture contained 2 μg. wild-type (5*a*) protein, or 27 μg. *het* C protein, or both.

Enzyme preparation	Temperature			
	22·0°	30·0°	21·8°	30·8°
	Reaction rate			
	Back reaction		Forward reaction	
Wild-type	48	76	8·0	13·0
Heterocaryon	45	92	9·5	22·4
Mixture	98	170	16·0	34·0

Michaelis constants

Michaelis constants for all five substrates were determined for partially purified preparations from wild-type and the two kinds of heterocaryons. The values, which, in the case of wild-type, are in reasonable agreement with those previously reported (Fincham, 1957), are shown in Table 5. Some variation is evident in the estimates for wild-type; some of this could be due to small differences in temperature and pH value. None of the differences between the heterocaryons and wild-type can be considered as outside the range of experimental variation, except the lower affinity for glutamate shown by the $am^1 + am^3$ preparations, and, possibly, the rather high affinity for ammonium ion shown by the $am^1 + am^2$ preparation. As a check on the difference in Michaelis constants for glutamate, wild-type and *het* A preparations were compared in the same experiment under conditions of identical phosphate concentration, pH, and temperature. The data, and those for separate experiments on *het* B and *het* C preparations, are shown in Fig. 3. There seems to be no doubt that the difference between $am^1 + am^3$, on the one hand, and

Table 5. *Michaelis constants for glutamic dehydrogenase preparations from* Neurospora crassa *wild-type and heterocaryons*

Except where otherwise indicated, the constants were determined at pH 8·0, and with the concentrations of the non-varying components of the reaction mixtures as in the standard assays systems

Strain	K_m (molar concentration × 10^5)				
	Glutamate	α-Ketoglutarate	NH_4^+	TPNH	TPN
Wild type ST*A* (2 preparations)	980 750	390 (1) 310 (1)	110 (2) 100 (2)	3 (1, 3) 3 (1, 2)	5 6
Wild type 5*a*	1100* (4)	—	—	—	—
$am^1 + am^2$ *het* D	900	320 (1)	50 (2)	3 (1, 2)	6
$am^1 + am^2$ *het* C	800* (4)	—	—	—	—
$am^1 + am^3$ *het* B	3000* (5)	420	100 (2)	3·5 (2)	4 (5)
$am^1 + am^3$ *het* A	2700* (4)	—	—	—	—

* See Fig. 3.

Notes. (1) NH_4Cl at 10 mM; (2) α-ketoglutarate at 12 mM; (3) α-ketoglutarate at 10 mM; (4) pH 7·9; (5) pH 7·85.

am^1+am^2 and wild-type, on the other, is significant. The possibility that am^1+am^3 preparations contained an inhibitor having the effect of reducing affinity for glutamate, or, conversely, that they lacked an activator present in wild-type preparations and having the opposite effect, receives no support from the experiments on mixed preparations shown in Table 6. While some degree of interaction is not ruled out, it is clear that neither type of preparation acquired the properties of the other in the mixtures. It thus seems simplest to suppose that am^1+am^3 heterocaryons produce a further distinct form of glutamic dehydrogenase.

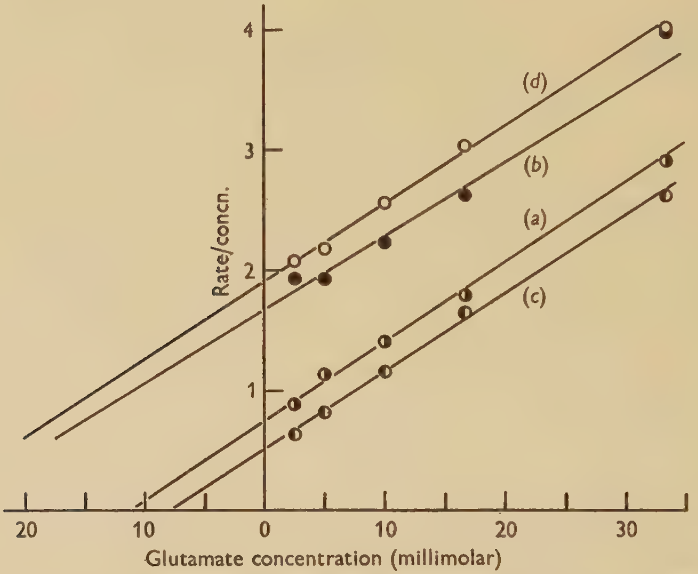

Fig. 3. Estimates of Michaelis constants for glutamic acid: (*a*) 4 μg. protein of wild-type 5*a*, 21·5°, pH 7·9 (K_m = 11 mM); (*b*) 14 μg. protein of am^1+am^3 *het* A, 21·5°, pH 7·9 (K_m = approx. 27 mM); (*c*) 68 μg. protein of am^1+am^2 *het* C, 22°, pH 8·0 (K_m = 8 mM); (*d*) 30 μg. protein of am^1+am^3 *het* B, 22°, pH 7·85 (K_m = approx. 30 mM).

Table 6. *Effect of glutamate concentration on rate of forward reaction with* Neurospora crassa *wild-type and heterocaryon* (am^1+am^3) *glutamic dehydrogenase preparations separately and in mixtures*

In Expt. 1: 13 μg. protein of wild-type (5*a*), or 38 μg. protein of *het* A, or both. In Expt. 2: 6 μg. protein of wild-type (2*a*) or 29 μg. protein of *het* A, or both. Temperature: 21° in Expt. 1; 22·5° in Expt. 2. pH of reaction mixtures: 7·85 in Expt. 1; 7·9 in Expt. 2.

	Expt. 1		Expt. 2	
	Glutamate concn. (mM)			
Enzyme preparation	3·3	33·3	5·0	33·3
	Reaction rate			
Wild-type	6·3	23·5	3·2	8·6
Heterocaryon	2·1	12·0	3·8	15·6
Mixture	8·2	33·0	7·9	26·4

DISCUSSION

From the foregoing data it seems highly probable that $am^1 + am^2$ and $am^1 + am^3$ heterocaryons both produce peculiar kinds of glutamic dehydrogenase. The $am^1 + am^2$ enzyme differs from wild-type enzyme in its capacity for activation by mild heat, and by its instability at higher temperatures, while the $am^1 + am^3$ enzyme is distinguished by its high Michaelis constant for glutamate. The nature of the differences between the various kinds of enzyme is, at present, unknown. The enzyme produced by $am^1 + am^2$ has, in its capacity for heat-activation, something in common with the enzyme produced by am^{2l}, an allele obtained from am^2 by treatment with ultraviolet light (Fincham, 1957; Fincham & Pateman, 1957*b*). In fact, enzyme from $am^1 + am^2$ is quite closely similar in its activity and response to heat to that from heterocaryons between am^{2l} and some other alleles, such as am^4 (Fincham, 1959). It seems likely that enzymes from all these sources share a rather flexible structure which tends to assume a more nearly 'correct' configuration at higher temperatures.

The facts that it is possible to represent complementation relationships between alleles by linear maps (Catcheside & Overton, 1959; Woodward *et al.* 1958; Fincham, 1959), and that, in at least one case (Giles, 1959), there seems to be a correlation between a complementation map and a chromosome map based on crossover frequencies, suggest that the complementation process involves interaction between linear structures with defects corresponding in position to the site of mutation within the chromosomal locus. One idea (Woodward *et al.* 1958) has been that cytoplasmic products of different alleles may undergo some kind of recombination process, analogous to crossing-over between chromosomes. Such a process should lead to the formation of a *wild-type* product in an amount proportional to the distance apart of the lesions in the two structures. The data of Woodward *et al.* (1958) for the Neurospora *ad*-4 locus show a tendency, which is perhaps not entirely convincing, for pairs of alleles represented as being relatively far apart on the complementation map to produce more enzyme (in this case adenylosuccinase) than pairs closer together. However, the fact that, in the cases of two pairs of *am* alleles, the interaction product seems *not* to be wild-type enzyme, tells against any hypothesis involving crossing-over or recombination. It seems more likely that complementation, at least in the *am* case, involves *combination* of allelic products, rather than recombination. The possibility of making complementation maps would still be understandable on this basis, since products with lesions in different places might be able to 'cover' each other's deficiencies, while products with similar or effectively overlapping lesions could not do so.

There is at present nothing to show whether the molecules directly involved in complementation are protein or components of the protein-forming system (perhaps ribonucleic acid). In view of the present results, however, the idea of complementation at the protein stage becomes attractive. It has been reported (Soyama, 1958) that animal glutamic dehydrogenase can be dissociated into at least eight subunits, which, since they retain enzyme activity, are probably identical. If the Neurospora enzyme is similar in this respect, it may be that each mutant *am* allele is responsible for the formation of a more or less defective subunit, and that subunits, which, in heterocaryons, may be derived from different alleles, become polymerized after synthesis. It is easy to imagine that two individually defective polypeptide chains could provide mutual support and form a 'correctly' folded protein through copolymerization. An attractive feature of this hypothesis, which is essentially the same as that advanced by Catcheside & Overton (1959), is that it is subject to experimental test, since, if it is true, it should be possible to demonstrate the complementation reaction *in vitro* under appropriate conditions. A model of the kind of experiment required is provided by the recent work of Singer & Itano (1959) on the dissociation and reassociation of haemoglobin subunits. Complementation *in vitro* has been demonstrated for certain pairs of *ad*-4 (adenylosuccinase-deficient) mutants of Neurospora (Woodward, 1959).

Considerable attention has been given in recent years to the question of the definition of genetic units. Benzer (1957) has proposed that the functional unit should be defined as the *cistron*. According to his definition, when two mutant genomes, introduced into the same cell, produce a defective phenotype, then the mutations are in the same cistron; when a non-defective phenotype results, the mutations are in different cistrons. The delimitation of cistrons is not always straightforward, however, since it depends on what one is prepared to accept as a non-defective phenotype. If ability to grow more or less normally on minimal medium is adopted as the criterion, then the phenomenon of inter-allele complementation means that several chromosomal loci in Neurospora, which appear to be units of function of some sort since they are concerned with single enzymes, have each to be subdivided into several cistrons. This rather unsatisfactory conclusion can be avoided, however, if one stipulates that the phenotype has to be non-defective at the *enzyme* level before two mutants are recognized as belonging to different cistrons. If the situation shown in the present paper is general in cases of inter-allele complementation, then all the recognized loci in Neurospora may, after all, be cistrons in this qualified sense.

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The Classification of Certain Filamentous Bacteria with Respect to their Chemical Composition

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SUMMARY: Upon the basis of cell-wall composition the oral bacteria *Leptotrichia dentium* and *L. buccalis* appear to form a natural generic group related to *Nocardia* and possibly *Lactobacillus.* An aerobic oral actinomycete of the type commonly called *Nocardia* has been shown to resemble closely the anaerobic pathogenic *Actinomyces* which parasitizes man. The cell-wall composition of *Streptomyces, Nocardia, Lactobacillus* and *Fusobacterium* strains is also reported.

The purpose of this study is to determine the relationship between the filamentous bacteria of the oral flora and between these and the comparable forms from other sources.

The oral parasite, *Leptotrichia dentium* (Baird-Parker & Davis, 1958; Davis & Baird-Parker, 1959) possesses characters that suggest possible relationship with both *Nocardia* and *Streptomyces.* Nevertheless, it does not resemble either of these genera so closely as to make classification with one of them unequivocal upon morphological grounds alone. At the same time, the diagnostic characters of *Streptomyces* and *Nocardia* overlap to some extent; some species of the latter, e.g. *N. asteroides,* produce aerial mycelium and spores of distinctly Streptomyces-like appearance (Gordon & Mihm, 1958) and the vegetative mycelium of Streptomyces commonly fragments to yield Nocardia-like elements (Bisset, 1957; Bradley & Anderson, 1958).

It has, on the other hand, been suggested that *Leptotrichia buccalis* is closely related to *Lactobacillus* upon metabolic grounds (Hamilton & Zahler, 1957).

Although *Leptotrichia dentium* more closely resembles the Actinomycetales in its morphology, it is physiologically similar to *L. buccalis* in many ways and partly for this reason, and partly for convenience, the two organisms currently constitute the ill-defined genus *Leptotrichia.*

Leptotrichia buccalis has also been frequently confused with *Fusobacterium* so that certain specific names in this genus [e.g. *F. dentium* (Hine & Berry, 1937); *F. plauti-vincenti* (Jackins & Barker, 1951)] are synonyms for *L. buccalis.*

It is apparent that the relationships of all these genera and species require to be re-examined.

A further problem is the occurrence in the mouth of aerobic Gram-positive filamentous bacteria usually classified as *Nocardia* spp. (Morris, 1954). Although some of these have been identified with previously described species, e.g. *Nocardia corallina, N. rangoonensis, N. mesenterica,* by Mandel, Levy & Wasserman (1957) their taxonomy has not been developed to any great extent. Such organisms have been confused with the microaerophilic *Actinomyces israelii* and it is probable that the *A. naeslundii* of Thompson & Lovestedt (1951) and

Garrod (1952) represents one of the commonest of these oral organisms. The group 3*b* of Onisi & Nuckolls (1958) also appears to consist of such organisms.

We have attempted to resolve some of these problems by supplementing cultural, morphological and metabolic studies with chromatographic analyses of cell walls according to the technique of Cummins & Harris (1956, 1958).

MATERIALS AND METHODS

See Table 1 for strains used and their origins. The soil strains of *Nocardia* were supplied by Dr R. B. Cain of this department.

The isolation and maintenance of *Leptotrichia dentium* and *L. buccalis* has been described elsewhere (Davis & Baird-Parker, 1959) and so also has the isolation and classification of *Lactobacillus* spp. (Davis, 1955), and the isolation of *Fusobacterium* spp. (Baird-Parker, 1957).

Table 1. *Strains examined*

Strain no.	Description	Source
SG	*Streptomyces griseus*	Old laboratory culture
S25	*Streptomyces* sp.	Soil
S26	*Streptomyces* sp.	Soil
600	*S. hortonensis*	NCTC
4523	*Actinomyces buccalis*	NCTC
8595	*Nocardia asteroides*	NCTC
C1	*Nocardia* sp.	Soil
C2	*Nocardia* sp.	Soil
2A1	*Leptotrichia dentium*	Human saliva
1C1	*L. dentium*	Human saliva
V1	*L. dentium*	Human saliva
LD	*L. dentium*	Human saliva
LB1	*Leptotrichia buccalis*	Human saliva
LB2	*L. buccalis*	Human saliva
LB3	*L. buccalis*	Human saliva
G5A	Aerobic actinomycete	Human saliva
T3	Aerobic actinomycete	Human saliva
4A	*Lactobacillus fermenti*	Human saliva
6G	*L. buchneri*	Human saliva
3D	*L. plantarum*	Human saliva
2A	*L. casei*	Human saliva
8C	*L. salivarius*	Human saliva
1A	*L. acidophilus*	Human saliva
F18	*Fusobacterium* sp.	Human saliva
FP	*Fusobacterium* sp.	Human saliva

Oral aerobic actinomycetes were isolated upon nutrient agar supplemented with 0·3% (w/v) yeast extract and 0·2% (w/v) soluble starch. This medium was also used for the maintenance of Streptomyces, Nocardia, and the NCTC strains.

Chromatographic analysis

Cell growth. All strains were grown in liquid media in bulk cultures. The aerobic organisms, i.e. Nocardia, Streptomyces, *Leptotrichia dentium*, oral aerobic actinomycetes, and NCTC strains were inoculated into Carrel type flasks containing 200 ml. of nutrient broth plus 1 % (w/v) glucose and 0·2%

(w/v) soluble starch, pH 7·4, in shallow layers. Soil bacteria were incubated at 28°, the rest at 37°, and the flasks shaken daily to distribute growth. Cells were harvested when good growth was evident, usually 3–5 days.

Lactobacillus strains were cultured in screw-capped bottles containing the following medium: vitamin-free casein hydrolysate 20 g., yeast-extract 5 g., sodium acetate 15 g., glucose 20 g., salts A 5 ml., salts B 5 ml., Tween 80 1 ml., distilled water 1000 ml., pH 7·0. (For salts A and B see Hayward, 1957.)

Inoculated bottles of medium were loosely closed, especially in the case of heterofermentative strains, to allow generated gas to escape.

Anaerobic organisms (*Leptotrichia buccalis* and Fusobacterium) were grown in tightly closed screw-capped bottles completely filled with Baird-Parker's (1957) medium, omitting the antibiotics and dye.

Cells were harvested by centrifuging, and washed three times in distilled water. (In some cases, e.g. Nocardia and Streptomyces, the alkaline ethanol treatment of Cummins & Harris (1958) was applied.) The cell mass was divided into two parts with normally the harvest of 200 ml. of medium in each.

Sugar extracts. One sample of cells was suspended in *c.* 5 ml. of 2 N-H_2SO_4 in a tightly closed 20 ml. screw-capped McCartney bottle, and heated for 4 hr. in a steamer. This whole cell hydrolysate was neutralized to pH 4·8 using freshly prepared saturated barium hydroxide solution and methyl red as indicator. Precipitated sulphate and cells were removed by centrifuging and the clear supernatant evaporated to dryness in vacuum over calcium chloride. The extract was re-suspended in *c.* 0·2–0·5 ml. 10% iso-propyl alcohol solution.

Amino acid extracts. The second sample of cells was made into a thick slurry with water and the cells broken by exposing them to high-frequency vibrations using a 'Mullard' 60 W. ultrasonic drill fed by an amplifier, supplying 180 W. at 20,000 kc./s.

The rate of cell breakage by this method varies greatly with different types of organism. Gram-negative organisms (Fusobacterium, *Escherichia coli*) break readily (1–2 min. exposure); Gram-positive filamentous bacteria (Streptomyces, Corynebacterium, etc.) require 20–30 min.; small Gram-positive bacilli (certain lactobacilli) and coccal forms are difficult to rupture, requiring 1 hr. or more. Broken cells were centrifuged, resuspended in 0·5% trypsin solution, and solid sodium bicarbonate added to produce *c.* pH 8·6. trypsin digestion was carried out at 37° for 12–18 hr.; the cell walls were centrifuged, washed three times in distilled water, and resuspended in *c.* 5 ml. 6 N-HCl. Hydrolysis was carried out in tightly closed screw-capped McCartney bottles by autoclaving at 25–30 lb./sq.in. pressure for 4 hr. The hydrolysate was filtered through sintered glass (porosity no. 4), evaporated to dryness on a steam bath, re-dissolved in 1–2 ml. 10% iso-propyl alcohol, one spot of concentrated ammonia added and the extract refiltered, evaporated dry and re-dissolved in *c.* 0·3 ml. 10% iso-propyl alcohol.

Chromatography. Amino acid and sugar extracts were analysed by paper chromatography using the descending, one-dimensional method, and Whatman no. 1 paper.

Each extract was run in two solvents.

Sugars: (*a*) Iso-propyl alcohol (160) + water (40).
(*b*) Phenol (80) + water (20).

Amino acids: (*a*) Iso-propyl alcohol (70) + acetic acid (10) + water (20).
(*b*) Phenol (80) + water (20) + 8 hydroxyquinoline (0·008 g./100 ml.) in an atmosphere of ammonia.

Owing to the varying concentrations of different extracts, it was found advantageous to run each extract at three or four concentrations upon the chromatogram. This was achieved by using a platinum loop to load the chromatogram, applying, for example, 2, 6 and 10 spots while gently heating with a hair-dryer to control the spot size.

Sugar chromatograms were air-dried, dipped in aniline hydrogen phthalate and heated at 105° C. for 5 min.

Amino acid chromatograms were sprayed with 0·25 % (w/v) ninhydrin in acetone and heated at 105° C. for 5 min.

The isomers of diaminopimelic acid were differentiated, as far as this is possible, using Hoare & Work's (1957) method.

Biochemical and cultural characters

It is commonly stated that biochemical and cultural characters are of little value in differentiating Gram-positive filamentous bacteria into definite groups. In view of the small numbers of strains here described we would not dispute this statement, but simply record the results of certain tests which seem to us to be of possible value in the characterization of the aerobic types we have studied (Table 2). The differentiation of *Leptotrichia buccalis* from *L. dentium* has been described (Davis & Baird-Parker, 1959) and the characteristics of *Fusobacterium* spp. will form the basis of a subsequent publication.

Casein digestion and Xanthine and Tyrosine dissolution tests were carried out as described by Gordon & Mihm (1956). Gelatin liquefaction was tested by the method of Frazier (1926). Routine methods were used for the remaining tests.

RESULTS

The results are set out in Tables 2–4. In recording the cell-wall patterns we have adopted an arbitrary method whereby the major components are scored as positives and the minor components as traces. Using the relatively simple chromatographic methods here described it is considered safest to allow taxonomic significance only to major components, and as can be seen in Table 3 this renders the classification of organisms into groups at generic level quite easy. Seven such groups were differentiated and their salient cell-wall characteristics are compared in Table 4. The analyses obtained for *Streptomyces*, *Nocardia* and *Lactobacillus* spp. agree in the main with those reported by Cummins & Harris (1956, 1958). It will be noted that most of the bacteria yield ribose upon sugar analysis; this is to be expected in view of the presence of ribonucleic acid in bacteria; it is unlikely, however, that any other sugar is

Table 2. *Selected physiological and cultural characters of various aerobic actinomycetes*

Strain	No. of strains	Catalase production	Nitrate reduction	Nitrite reduction	Gelatin liquefaction	Starch hydrolysis	Casein digestion	Hippurate hydrolysis	Tyrosine dissolution	Xanthine dissolution	Final pH in 1 % glucose broth	Growth temperature	Growth in glucose broth
Soil streptomyces	3	+	–	–	+	+	+	–	W	–	7·5	28°	flocculent
Streptomyces hortonensis NCTC 600	1	+	+	–	+	+	W	–	–	–	7·5	better at 28°	granular
Actinomyces buccalis NCTC 4523	1	+	–	–	+	+	+	–	+	W	7·5	28 or 37°	granular
Soil Nocardia	3	+	–	–	–	W	–	W	+	–	8·3	28°	smooth turbidity
Nocardia asteroides	1	+	+	–	–	W	–	–	–	–	8·0	37°	granular/flocculent
Leptotrichia dentium	4	+	+	–	–	+	–	+	–	–	5·2	37°	granular
Aerobic oral actinomycetes	8	+	+	+	+	W	–	–	+	–	5·2	37°	smooth turbidity

+ = positive, W = weakly positive, – = negative.

Table 3. *Cell-wall composition of strains studied*

Group	Strain	LL-Diamino-pimelic acid	DL-or DD-Diamino-pimelic acid	Lysine	Glutamic acid	Alanine	Glycine	Serine	Leucine	Aspartic acid	Slow runner	Arabinose	Ribose	Glucose	Galactose	Rhamnose	Unidentified hexosamine
Streptomyces	*S. griseus*	+	–	–	+	+	+	–	–	–	–	–	Tr.	Tr.	–	–	Tr.
	S25	+	–	Tr.	+	+	+	Tr.	Tr.	–	–	–	Tr.	Tr.	–	–	–
	S26	+	–	Tr.	+	+	+	–	Tr.	Tr.	–	–	Tr.	Tr.	–	–	Tr.
	NCTC 4523	+	–	–	+	+	+	–	–	Tr.	–	–	–	–	–	–	–
	NCTC 600	+	–	–	+	+	+	–	–	–	–	–	Tr.	+	–	–	–
Nocardia	C1	–	+	–	+	+	Tr.	–	Tr.	–	–	+	–	–	+	–	Tr.
	C2	–	+	–	+	+	Tr.	–	Tr.	–	–	+	–	–	+	–	–
	NCTC 8595	–	+	–	+	+	Tr.	–	Tr.	–	–	+	–	–	+	–	–
Leptotrichia dentium	2A1	–	+	Tr.	+	+	Tr.	–	Tr.	–	–	+	Tr.	+	–	–	Tr.
	1C1	–	+	Tr.	+	+	Tr.	–	Tr.	–	–	+	Tr.	+	–	–	Tr.
	V1	–	+	Tr.	+	+	–	Tr.	Tr.	–	–	+	Tr.	+	–	–	Tr.
	LD	–	+	–	+	+	Tr.	–	–	–	–	+	Tr.	+	–	–	Tr.
Leptotrichia buccalis	LB1	–	+	Tr.	+	+	Tr.	Tr.	Tr.	–	–	–	Tr.	+	–	–	–
	LB2	–	+	–	+	+	Tr.	–	Tr.	Tr.	–	–	Tr.	+	–	–	–
	LB3	–	+	Tr.	+	+	+	–	Tr.	–	–	–	Tr.	+	–	–	–
Oral aerobic actinomycetes	G5A	–	–	+	+	+	Tr.	–	Tr.	Tr.	–	–	Tr.	–	+	–	Tr.
	T3	–	–	+	+	+	Tr.	–	Tr.	Tr.	–	–	Tr.	–	+	–	Tr.
Lactobacillus	*L. fermenti*	–	–	Tr.	+	+	Tr.	–	Tr.	Tr.	–	–	Tr.	+	–	–	Tr.
	L. buchneri	–	–	+	+	+	Tr.	–	Tr.	–	–	–	Tr.	+	–	–	–
	L. salivarius	–	–	+	+	+	Tr.	–	Tr.	–	–	–	Tr.	+	Tr.	–	Tr.
	L. acidophilus	–	–	+	+	+	Tr.	Tr.	Tr.	Tr.	–	–	Tr.	+	–	Tr.	Tr.
	L. casei	–	+	Tr.	+	+	Tr.	Tr.	Tr.	Tr.	–	–	Tr.	+	Tr.	+	Tr.
	L. plantarum	–	+	Tr.	+	+	Tr.	–	Tr.	Tr.	–	–	Tr.	+	Tr.	–	Tr.
Fusobacterium	F18	–	–	+	+	+	+	+	+	+	+	–	Tr.	+	Tr.	–	Tr.
	FP	–	–	+	+	+	+	+	+	Tr.	+	–	Tr.	+	Tr.	–	Tr.

+ = major component, Tr. = minor component, – = not detected.

Table 4. *Comparative cell-wall patterns of certain groups and genera of bacteria*

	LL-Diamino-pimelic acid	DL or DD-Diamino-pimilic acid	Lysine	Glutamic acid	Alanine	Glycine	Serine	Leucine	Aspartic acid	Slow runner	Arabi-nose	Glucose	Galac-tose
Streptomyces	+	−	−	+	+	+	−	−	−	−	−	−	−
Nocardia	−	+	−	+	+	−	−	−	−	−	+	−	+
Leptotrichia dentium	−	+	−	+	+	−	−	−	−	−	+	+	−
L. buccalis	−	+	−	+	+	−	−	−	−	−	−	+	−
Oral aerobic actinomycetes	−	−	+	+	+	−	−	−	−	−	−	−	+
Lactobacillus	No set generic pattern												
Fusobacterium	−	−	+	+	+	+	+	+	+	+	−	+	−
*Actinomyces israelii**	−	−	+	+	+	−	−	−	−	−	−	−	+

* From Cummins & Harris, 1958.

commonly present in large amounts in the cytoplasm or nucleus and the non-ribose sugars detected in whole cell hydrolysates may be assumed to be cell-wall components. We have compared sugar analyses of whole cells and cell walls in a few representative strains and found them to be identical.

Amino sugars, which appear to occur almost universally in bacterial cell walls, were not readily detected by the methods here described. They could not be picked out upon the amino acid chromatograms, but were often noticeable in the sugar runs. However, the latter method does not allow their clear differentiation in most cases and spots representing glucosamine, galactosamine or muramic acid are therefore recorded under a common heading as hexosamines (Table 3).

DISCUSSION

The classification of bacteria must depend upon information obtained by the use of various techniques. Chemical analysis of bacterial cellular components appears to be a useful method of confirming taxonomic groupings and in some cases clarifying relationships, which may be otherwise obscure, between certain bacteria.

In the present study we have tried to keep the technical work within the scope of the non-specialist and extract the maximum of useful information by the simplest means, hence the use of whole cell hydrolysates for sugar analyses and one-dimensional chromatography. The questions posed in the introduction to this paper may be answered as follows:

Leptotrichia dentium resembles the genus *Nocardia* in cell-wall amino-acid pattern, but differs from it in sugar pattern. No relationship with the genus *Streptomyces* is indicated.

Leptotrichia dentium and *L. buccalis* exhibit the same amino acid patterns but differ by the absence of arabinose in the sugar component of *L. buccalis*. Whether these results indicate that the two organisms may be regarded without doubt as two species of a single genus depends upon the taxonomic significance placed upon the amino-acid and sugar patterns respectively. It has been suggested that the amino-acid patterns characterize genera and sugar patterns species, but too little evidence is yet available to prove this idea. In the *Nocardia–Corynebacterium–Mycobacterium* group of genera there appears to be little variation in either pattern between different species or even genera (Cummins & Harris, 1958). Although lacking final proof it therefore seems reasonable to retain the genus *Leptotrichia* as distinct from, but closely related to, the genus *Nocardia* and to retain *L. dentium* and *L. buccalis* as its two species.

Among the lactobacilli, *Lactobacillus plantarum* and *L. casei* show cell-wall patterns grossly similar to *Leptotrichia buccalis* in both amino acids and sugars. In contrast to such groups as *Nocardia–Mycobacterium* referred to above, there is considerable variation of pattern between species of the genus *Lactobacillus* and upon the basis of cell-wall pattern and physiology the inclusion of *Leptotrichia buccalis* as a species of the genus *Lactobacillus* would not appear anomalous. Morphologically, however, no *Lactobacillus* sp. resembles the distinctive *Leptotrichia buccalis* form, nor is it feasible to place *L. dentium* with the lactobacilli on morphological or physiological grounds. The question thus resolves into the choice between placing *L. buccalis* in the genus *Lactobacillus* and *Leptotrichia dentium* in the genus *Nocardia*, with neither grouping being perfect, or retaining the genus *Leptotrichia* as suggested above. *Fusobacterium* spp. have a cell-wall pattern distinguished by its relative complexity and the presence of a distinctive slow running component of unknown composition. Amino acid complexity is characteristic of the cell walls of Gram-negative species. The slow running component runs slower than diaminopimelic acid in the iso-propyl alcohol solvent, and in Hoare & Work's solvent it can be separated into two slow running spots.

The Nocardia-like aerobic actinomycetes isolated from the human mouth for this study are not classifiable as members of the genus *Nocardia* on the evidence of cell-wall patterns. They lack both diaminopimelic acid and arabinose, and upon the results so far available appear to have a cell-wall composition very like the true, microaerophilic Actinomyces of human origin (Cummins & Harris, 1958). If this classification should prove correct the definition of the genus *Actinomyces* will require modification to include aerobic non-pathogenic organisms of this type.

It is interesting to note that the soil Nocardia strains are identical in cell-wall composition with *Nocardia asteroides*, which is morphologically quite different from them in that it produces aerial chains of spores. The only *Nocardia* spp. investigated by Cummins & Harris (1958) which was not of animal origin, was *N. gardneri* and this turned out to be a Streptomyces. Although *Nocardia* is predominantly a saprophytic genus, the atypical animal strains are commonly used as examples of the group; our results show that *Nocardia* spp. from saprophytic and parasitic sites, differing widely in morphological potential, nevertheless form a cohesive group in terms of cell-wall composition.

Upon biochemical as well as cell-wall characters the NCTC strain 4523 called *Actinomyces buccalis* should be re-classified with *Streptomyces*.

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The Bacteriophages of *Clostridium perfringens*

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SUMMARY: A search has been made for phages active on *Clostridium perfringens*. types A, B, C, D, E, and F. Twelve of 49 type A strains, 10 of 31 type B strains, 10 of 26 type C strains and none of either 38 type D, 5 type E or 3 type F strains were lysogenic. The 'temperate' phages obtained from these lysogenic cultures, together with similar phages obtained from crude material, such as sewage, were only active on strains of the same type as the lysogenic culture from which they were isolated; some of them were very host-specific. It was possible to induce partial lysis in lysogenic strains by ultraviolet radiation, nitrogen mustard and mercaptoacetic acid.

Other phages isolated from crude material resembled the 'virulent' phages of aerobic bacteria. These phages lysed strains belonging to types A, B, C, D, and F.

Despite a long search for phages that would lyse them, a high proportion of the strains of *Clostridium perfringens* examined remained insusceptible to all the phages isolated. A close relationship between phage susceptibility and colonial morphology was noted. Smooth and rough strains were usually phage susceptible; mucoid strains were usually phage-resistant.

Bacteriophages which lyse anaerobic bacteria have been studied much less than those active on aerobic bacteria. Recently, however, the position has improved as far as phages active on *Clostridium perfringens* are concerned, principally by the studies of Guelin and her collaborators (Elford, Guelin, Hotchin & Challice, 1953; Guelin, 1949, 1950, 1955; Guelin & Kreguer, 1950; Kreguer, Guelin & Le Bris, 1947). Although these workers have commented upon the selective action of some of the phages which they isolated from crude materials, such as polluted water, little published information exists as to the specificity of phages for the different types of *C. perfringens*. Also, no work appears to have been published upon the existence and behaviour of lysogenic cultures of these bacteria. The present studies were undertaken principally in order to obtain information on these two points.

METHODS

Clostridium perfringens *cultures*. The studies were mainly concerned with 152 strains. Of these, 49 belonged to type A, 31 to type B, 26 to type C, 38 to type D, 5 to type E and 3 to type F. Twelve of the type A strains and the 3 type F strains had been responsible for outbreaks of food poisoning; the remainder had been isolated from man and a variety of domestic animals. The majority of the B, C, D and E strains had originated from outbreaks of disease in sheep. Freshly isolated strains were also used from time to time for different purposes; apart from the fact that they produced lecithinase, no information was sought as to what type they belonged.

Solid medium. The solid medium used for all the studies consisted of blood agar base (Oxoid), 40 g.; bacto-casitone (Difco), 10 g.; yeast extract (Oxoid), 2·0 g.; distilled water to 1000 ml. This was found to be the most suitable after studying a variety of media. Some ingredients were definitely disadvantageous. The addition of glucose, for example, yielded bacterial growth which, although more prolific, possessed an uneven surface which made plaques difficult to discern.

Liquid media. Although several different liquid media were used in searching for phage in crude material, cooked meat medium (Oxoid) in 1 oz. screw-capped bottles was used for all other purposes.

Phosphate buffer. This consisted of KH_2PO_4, 3·4 g.; Na_2HPO_4, 6·0 g.; distilled water, 1000 ml. Its pH was 7·0.

Search for lysogenic cultures. Modifications of the cross-culture technique (Fisk, 1942) were used to identify lysogenic strains. Cultures were studied in batches of 20, various combinations being employed until most of the 152 strains had been tested against each other. The 20 strains were grown in cooked meat medium for 18 hr. at 37°; each was then used as an indicator strain by spreading 5 drops (0·1 ml.) of the culture over the surface of a dry plate of solid medium. When dry, the remaining 19 cultures were 'spotted' on to it. The plates were incubated for 18 hr. at 37° in an atmosphere of 95 % (v/v) hydrogen + 5 % (v/v) carbon dioxide. Phage action was revealed by the presence of zones of lysis or by discrete plaques. The interpretation was sometimes complicated by the fact that many of the strains produced inhibitory substances for each other. These substances were distinguished from phage by their failure to reproduce in cultures of the indicator strain.

Several modifications of the technique described above were also used, e.g. liquid cultures of different ages and incubation of the tests at different temperatures from 37° to 28°. Different media were also used and a useful expedient was to add nitrogen mustard to the cooked meat medium because it was found to partially 'induce' phage production in lysogenic cultures of *Clostridium perfringens.* Supernatant fluids of liquid cultures after centrifugation were also examined as well as cultures in which the bacteria had been killed either by heating to 58° for 30 min. or by chloroform (Fredericq, 1950). On other occasions, cultures were incubated, before testing, with a strain known to be susceptible to phage previously isolated. On one occasion oxytetracycline-resistant cultures were used as indicator strains growing on a medium containing oxytetracycline hydrochloride, 1 μg./ml.

The isolation of phages from crude material. Several methods were used in attempts to isolate phages from material such as sewage, pond and river water, faeces of healthy animals and intestinal contents of sheep dead from *Clostridium perfringens* type D infection; no one method showed itself to be uniformly the best. Crude material was added to various liquid media and incubated for 6–24 hr. at temperatures between 28° and 37°. Sometimes the material was heated to 58° for 30 min. before incubation and sometimes streptomycin sulphate (20 μg./ml.), was added after heating. On other occasions, cultures, particularly those which had not at that time been found to be susceptible to the phages already isolated, were also added to the medium before incubation. After incubation, the cultures were centrifuged and the supernatant fluids, before and after heating to 58° for 30 min. or treating with chloroform, were spotted on to plates spread with different indicator cultures of *C. perfringens*. The plates were incubated at 28° or 37° anaerobically for 18 hr. and then examined for phage action. Sometimes the crude material was centrifuged and filtered through a Seitz E.K. sterilizing pad before it was incubated with the selected cultures in liquid media.

Purification and propagation of phages. When phage action was noted the lytic area was picked and mixed, in different dilutions, with a meat broth culture of the susceptible strain; the mixtures were then spread over the surface of a plate of solid medium and incubated anaerobically at 37° for 18 hr. (The word 'strain' is used throughout with reference to bacteria and not to phages.) A discrete plaque was picked and replated with the propagating strain. This process was repeated twice. Finally, a discrete plaque with some of the surrounding bacterial growth was picked into meat broth and incubated at 37° until lysis occurred. Usually, more of the susceptible strain was then added and incubation continued. Clearing of the culture was conveniently followed by comparison with a control culture inoculated with the propagating strain only. Finally, the lytic material was centrifuged, filtered through a Seitz E.K. sterilizing pad and tested for bacterial sterility. By this process it was usually possible to obtained relatively high titred phage preparations (C.T.D. of 10^{-3}–10^{-6}), the potency of the preparation being only slightly reduced by filtration. Known lysogenic strains were never used as propagating strains.

Titration of phage preparations. A meat broth culture of the propagating strain of *Clostridium perfringens* was spread evenly over the dried surface of a plate of solid medium, allowed to dry and then spotted with one drop (*c.* 1/150 ml.) of each tenfold dilution of the phage preparation in phosphate buffer. The plate was then incubated anaerobically for 18 hr. The highest dilution which produced semi-confluent plaques was chosen as the critical test dilution (C.T.D.), i.e. the dilution to be used routinely in comparing the activity of phages against different strains of *C. perfringens*. For this purpose, meat broth cultures of strains under test were spread over plates of solid media in the manner described above and the phages in C.T.D. strength on their original indicators spotted on to them before incubating at 37° for 18 hr. The particular definition of C.T.D. used in these studies was selected because it was unlikely to be confused with the activity of the antibacterial substances described above which some of the phage preparations contained.

Phage counts were carried out in a similar manner as the phage titrations, the technique being a modification of the Miles & Misra (1938) method for counting bacteria. When the phage content of lysogenic cultures was being determined, the cultures were first destroyed by heating to 58° for 30 min. or treating with chloroform; the few spores that remained did not complicate the phage-counting procedure.

Preparation of phage-resistant strains of Clostridium perfringens. Secondary colonies appearing in areas of complete lysis were picked and purified by three serial single colony isolations. They were then tested on solid medium to confirm their complete resistance to the undiluted phage preparation. Attempts to produce phage-resistant strains were sometimes made by prolonged incubation of a susceptible culture and phage in meat broth medium and then examining colonies obtained by plating on solid medium.

Cross resistance tests. These were performed by a modification of Bail's (1923) original method. Variants resistant to each of the phages were isolated from a single bacterial strain. The phages were then titrated against each of the variants.

Bacterial counts. These were carried out by the method of Miles & Misra (1938).

Determination of colonial morphology. Meat broth cultures were streaked on to plates of the solid medium and their colonial morphology was then determined according to the method of Stevens (1935).

RESULTS

The incidence of lysogenic strains among different types of Clostridium perfringens. The incidence of demonstrably lysogenic strains is shown in Table 1. Phages isolated from different strains have been assumed to be identical if they resembled each other in host range, plaque morphology, resistance to heat and chloroform, and behaved similarly in cross-resistance tests.

Table 1. *The incidence of lysogenic cultures of* Clostridium perfringens

Type of *C. perfringens*	No. of strains examined	No. lysogenic	Incidence of different phages
A	49	12	7 with A7, 5 with A8
B	31	10	All with B1
C	26	10	5 with C1, 1 with C1 and C4, 1 with each of C2, C3, C4 and C5

The susceptibility of strains of Clostridium perfringens *to phages isolated from crude material and from lysogenic cultures.* Table 2 shows the response of strains of *C. perfringens* to the phages isolated from the above lysogenic cultures as well as to phages isolated from crude material. The latter were of two kinds: six which lysed some type A strains only (and were therefore designated A phages, numbers 1–6), and 30 which also lysed strains of other types. These 30 were designated group phages. All the group phages had the same host range which was very wide although they differed in plaque morphology. On the other hand, all the A phages and also those isolated from type C strains were highly specific, some being active against only one strain of *C. perfringens* (e.g. C.1 in Table 2). The only B phage isolated attacked 18 of 31 type B strains tested; 10 of the insusceptible 13 type B strains were demonstrably lysogenic with this phage.

No strain of *Clostridium perfringens* could be lysogenized by the group phages. Some type A strains, however, could be lysogenized by the A phages

1–6 isolated from crude material, a fact which indicated that these phages had probably originated from lysogenic *C. perfringens* present in that material.

The relationship between phage susceptibility and colonial morphology. Apart from a few strains that were probably intermediate in character, those that had been phage typed could be classified, on the basis of colonial morphology, into mucoid, smooth and rough. The results of comparing colonial morphology and susceptibility to the group phages is illustrated in Table 3. With few exceptions, the smooth and rough strains were susceptible to these phages and the mucoid strains were resistant. The exceptions were mainly type C strains, 4 of the 14 mucoid strains referred to in Table 3 being phage susceptible; the phages, however, produced much smaller plaques on these 4 strains than on smooth strains.

Table 2. *The susceptibility of strains of* Clostridium perfringens *to phages*

Type of *C. perfringens*	No. of strains	Susceptibility to														
		Group phages	A phages								B phage	C phages				
			1	2	3	4	5	6	7	8	1	1	2	3	4	5
A	5	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−
	2	+	+	−	+	+	−	−	−	−	−	−	−	−	−	−
	1	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−
	1	+	−	−	−	−	+	−	+	−	−	−	−	−	−	−
	1	+	+	−	−	+	−	−	−	+	−	−	−	−	−	−
	1	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
	2	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
	2	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
	1	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
	1	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	32	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
B	18	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−
	13	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
C	1	+	−	−	−	−	−	−	−	−	−	+	+	+	+	−
	2	+	−	−	−	−	−	−	−	−	−	−	+	+	+	−
	4	+	−	−	−	−	−	−	−	−	−	−	+	+	−	−
	1	+	−	−	−	−	−	−	−	−	−	−	+	−	−	+
	1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
	8	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	9	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D	6	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	32	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
E	5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
F	1	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

+ = lysis; − = no action.

The group phages and A 1, 2, 3, 4, 5 and 6 were isolated from crude material; the remainder were isolated from lysogenic cultures.

A similar situation existed amongst freshly isolated strains. Of 12 strains tested immediately after their primary isolation from faeces of farm animals and human beings, 11 were mucoid and phage-resistant and one was smooth and phage-susceptible.

The relationship between phage susceptibility and colonial morphology, although definite, was less close in the case of the A and C phages, a few of the 19 mucoid A strains shown in Table 3, for example, being susceptible to the type A phages.

Varying the incubation temperature, the constitution of the culture medium and the density of the bacterial inoculum did not render the mucoid strains susceptible to phage action.

Table 3. *The relationship between colonial morphology and susceptibility to the group phages*

Type of *C. perfringens*	Colonial morphology	No. of strains tested	No. susceptible to group phages
A	Mucoid	19	0
	Smooth	6	6
	Rough	1	1
B	Mucoid	13	0
	Smooth	15	15
	Rough	3	2
C	Mucoid	14	4
	Smooth	4	4
	Rough	1	1
D	Mucoid	11	0
	Smooth	3	3
	Rough	2	2

Variation in colonial morphology and phage susceptibility. Several of the strains referred to above yielded both mucoid and smooth colonies when cultured on the solid media, one or other of these two variants being predominant in each strain; the smooth variants were susceptible to the group phages but the mucoid ones were not. During 10 serial single colony subcultures on the solid medium these mucoid and smooth variants bred true as regards colonial morphology and phage susceptibility. However, continuous subculture of the smooth variants, as well as of strains initially classified as smooth, in meat broth instead of on the solid medium, often gave rise to mucoid variants which were resistant to the group phages. As passage proceeded, these variants became more predominant and then, not infrequently, the only kind found in the meat broth. Passage of mucoid strains in meat broth gave rise only infrequently to smooth phage-sensitive variants. With only one of 10 mucoid strains did this form of variation occur. The smooth variants arose in small numbers only in cultures of this strain, remained for several passages and then disappeared.

The relationship between phage susceptibility and colonial morphology was particularly interesting among the 31 *Clostridium perfringens* type B strains studied. Of these, 18 were susceptible to both the group phages and phage B1 and 13 were resistant to them. None of the 18 susceptible strains but the majority of the 13 resistant strains were demonstrably lysogenic, the phage with which they were infected being, in all cases, B1. Consequently, it was thought at first that this lysogenic relationship was responsible for the 13 strains being resistant to the group phages as well as to B1. The fact that resistant variants prepared by lysogenizing susceptible strains with B1 were also resistant to the group phages supported this view. However, these resistant variants and the 13 resistant strains were all noted to possess a mucoid colonial morphology, whereas the 18 susceptible strains were either smooth or rough. Furthermore, serial subculture of the susceptible strains in meat broth often gave rise to mucoid variants which were resistant to the group phages but still susceptible to B1. It was probable, therefore, that the 13 strains were resistant to the group phages because they were mucoid and not because they were lysogenic with B1.

Characteristics of lysogenic cultures of Clostridium perfringens. The number of phage particles found in lysogenic cultures varied from strain to strain; it was usually small. The ratio between the numbers of viable bacteria and phage particles in a meat broth culture of a strain lysogenic with A1 was in the region of 100,000:1, with C1 was 20,000:1 and with B1 was 1000:1.

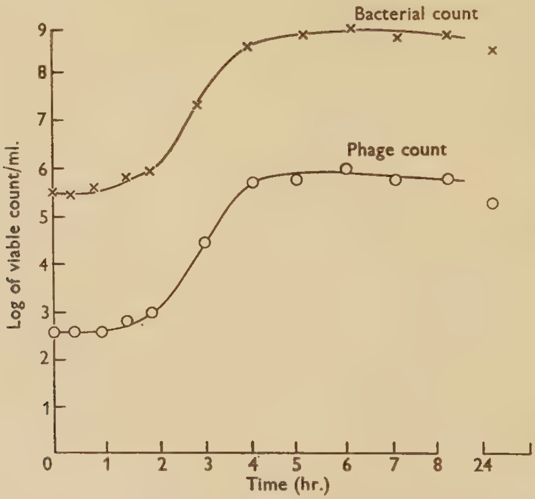

Fig. 1 The numbers of viable bacteria and phage particles in a growing culture of *Clostridium perfringens*, type B.

A typical growth curve of the bacteria and phage particles in a culture latently infected with B1 is shown in Fig. 1. The number of viable phage particles present at any time was directly related to the bacterial count.

Table 4 indicates that lysis could be partially inducted in lysogenic cultures by ultraviolet (u.v.) radiation, nitrogen mustard and to a much lesser extent by mercaptoacetic acid. The latter two substances were simply added to the meat broth medium in which the lysogenic cultures were being incubated; with u.v. irradiation, lysogenic bacteria suspended in distilled water were exposed and then incubated in meat broth medium. Treatment with urethane, colchicine or hydrogen peroxide had no effect on the liberation of phage particles by lysogenic cultures.

Apart from differences in host range and plaque morphology, some of the phages carried by lysogenic strains differed markedly from each other in regard to resistance to heat and chloroform. For example, phage A7 resisted heating to 58° and chloroform treatment but phage A8 was killed by both. Phage B1 was killed by chloroform but not by heat treatment; the reverse was true with phage C1. These phages, too, differed in the time they remained viable at 3°.

Table 4. *The induction of lysis in lysogenic cultures of* Clostridium perfringens

	Inducing factor* for cultures latently infected with phage		
Substance	A7	B1	C1
Ultraviolet radiation	20	100	20
Nitrogen mustard	100 (0·04)	40 (0·01)	10,000 (0·01)
Mercaptoacetic acid	1 (0·5–8)	5 (4)	10 (4)

* This was the factor by which the number of phage particles liberated by a lysogenic culture under the influence of an inducing substance was greater than that liberated, at any period, by a control culture.

The figures in parentheses are the concentrations of inducing substance in mg./ml. that produced the record-inducing effect.

DISCUSSION

It is clear from the present investigation that the host-parasite relationships existing between phages and *Clostridium perfringens* closely resemble those that apply in the case of the aerobic bacteria. Such variations in plaque morphology and in resistance to heat and chloroform occurring in *C. perfringens* phages have been described in phages of aerobic bacteria. It is apparent, too, that the group phages with their inability to enter into the lysogenic state with bacteria fit the classification 'virulent' as applied to certain phages of aerobic bacteria. The others which were much more specific as regards host range and formed lysogenic cultures with appropriate bacteria resemble the 'temperate' phages that infect aerobic bacteria. The ability of u.v. radiation, nitrogen mustard and mercaptoacetic acid to induce lysis in lysogenic cultures is also a well-known feature of the 'aerobic' phages.

Although it was possible to identify the type of *Clostridium perfringens* to which some strains belonged by the use of the type-specific phages, such a procedure would probably not be of much practical value because of the large proportion of strains that were insusceptible to the action of all the phages (61·2 %). An exception may be made in the case of type B because all except a few of the strains of this type examined were either susceptible to phage B1 or could be easily shown to be latently infected with this phage.

Despite the exhaustive nature of our search for phages, it was surprising that so many strains of *Clostridium perfringens* remained insusceptible to all the phages isolated. This was undoubtedly associated with the fact that many of these strains were mucoid. The close relationship in the type B strains between lysogenicity with phage B1 and mucoid colonial morphology is interesting. It is not inconceivable that in nature, infection of susceptible smooth strains with phage B1 may play a part in the evolution of mucoid forms of type B strains.

The relationship between colonial morphology and phage susceptibility, although close, was not complete, a few of the A phages in particular being active on certain mucoid strains. In view of this, it must be borne in mind that the use of more suitable indicator strains than those employed in the present investigation might reveal many more strains to be lysogenic, a state of affairs that might alter the whole outlook for the phage typing of *Clostridium perfringens*.

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Rapid identification of *Bacillus anthracis* by Microscopical Observation of Bacteriophage Lysis

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SUMMARY: When young growing filaments or microcolonies of *Bacillus anthracis* were treated with specific bacteriophage, fragmentation of the filaments followed by complete disintegration of the microcolony were visible microscopically within 2–3 hr. Other Bacillus species were unaffected by the phage. This technique is a simple one for accelerating the identification of *Bacillus anthracis*.

Bacillus anthracis may be distinguished from the closely related *B. cereus* by its susceptibility to a specific bacteriophage (McCloy, 1951). The usual technique for testing a suspected strain with phage consists in spotting the undiluted phage on a bacterial lawn on agar, and observing for lysis after incubation overnight (McCloy, 1951; Brown & Cherry, 1955). It seemed that identification could be accelerated by applying the phage to the bacteria at an earlier stage of their growth, and observing lysis microscopically. The experiments reported in this paper confirmed this prediction.

METHODS AND RESULTS

Bacteriophage. The bacteriophage used was a virulent mutant α of a temperate phage β, derived from a *Bacillus cereus* strain W (McCloy, 1958). The phage was used undiluted at a titre of $1{\cdot}5 \times 10^{10}$ particles/ml. The phage may be propagated on any strain of *B. anthracis*.

Propagation technique. A 10^{-2} dilution in broth of an overnight culture of *Bacillus anthracis* was incubated for 3 hr. at 37°. The culture tube was held in a Perspex drum rotating at about 2 rev./sec., inclined at 45° and fixed in a wooden box, the temperature inside which was controlled thermostatically. The drum was rotated by a low-power motor. The source of heat was a 100 W. electric light bulb placed beneath a metal reflector. To the resulting logarithmic phase culture was added α phage in the proportion of 10^9 particles/10 ml. culture, and incubation continued until clearing was complete, usually about $2\frac{1}{2}$ hr. after addition of the phage. The bacterial debris was centrifuged, and the supernatant liquid treated with 1 ml. chloroform. The lysate was left in contact with chloroform for 30 min. on the bench, and the chloroform then removed by suction. A sterility test was performed on the lysate, which was afterwards ready for use.

Technique of the phage-lysis microtest. Light inocula of *Bacillus anthracis* spores were allowed to germinate on plates of nutrient agar, with or without 5% (v/v) peptic extract of sheep blood (PSB) added. After 4–5 hr. incubation at 37°, single strand filaments of *B. anthracis* were visible under a binocular dissecting microscope, or a monocular microscope with transmitted light and 25 mm. or 16 mm. objectives. The filaments elongated rapidly in the early phase of growth, frequently reaching a length of 250–500 μ in one dimension before showing any tendency to form loops or folds. A single loop near the mid-point was, however, often characteristic. Plate 1, fig. 1, shows typical filaments of *B. anthracis* growing on nutrient agar inoculated 4 hr. previously with a light spore suspension. Plate 1, fig. 2, shows a microcolony of the same strain after growth for 6 hr. on nutrient agar.

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The position of the filament on the agar surface was marked with a straight wire. A small loopful of undiluted α phage was applied to the area of agar carrying the filament, and allowed to soak into it. There was some advantage in watching the application of the phage through the microscope to note any mechanical disturbance of the filament. It was also desirable to examine the treated area microscopically after the liquid had dried in, to ensure that the filament had not been removed accidentally on the loop. The risk of this happening was small.

The plate was returned to the 37° incubator for about 1 hr. At this time, and on several occasions during the subsequent hour, the treated area was examined microscopically for evidence of lysis in the filament. Between examinations the plate was returned to the incubator or kept in a hot-box (37°) equipped with a microscope. During the second hour after application of α phage, breaks appeared in the filament, due to the action of phage, and at about the end of this second hour, the filament had been completely lysed, and was represented by a thin groove in the agar corresponding to the position of the original filament (Pl. 1, figs. 3, 4). If the phage was applied to a filament at a slightly later stage of development than that shown in fig. 1, or to a microcolony such as that shown in fig. 2, then growth continued for another 1–1½ hr. before fragmentation began. Disintegration of the microcolony was, however, complete within 3 hr. of application of the phage.

Optical apparatus. The lytic changes were observed by using a monocular microscope with 16 and 4 mm. objectives and ×10 ocular, the source of illumination being transmitted light from a high-power filament lamp. The vertical illumination method described by Pearce & Powell (1951) was also tried. Observations were made with (i) a 16 mm. objective, using a light background; (ii) a 4 mm. objective, using a dark background.

Under the 16 mm. objective, filaments of *Bacillus anthracis* showed alternating light and dark areas due to parts of the filament being embedded in the agar while other segments lay flat on the surface. During the action of the phage, the grooves left in the agar by the lysed portions of the filaments contrasted strongly with the segments not yet lysed. But vertical illumination with this objective was not suitable for observation of fine detail, because of the 'frosted' appearance of the agar after application of the phage. By transmitted light the background was relatively clear.

The dark background for use with the 4 mm. objective was provided by incorporating nigrosin 0·5% (w/v) in the medium. When bacterial growth and phage lysis were allowed to take place on this medium, examination with the 4 mm. objective revealed very striking patterns of lysis. The irregular distribution of damage by the phage was very obvious, and when observation was continuous, individual cells could be seen to 'explode' as lysis took place.

Reproducibility of phage-lysis with different strains of Bacillus anthracis. Microcolonies of 13 virulent *B. anthracis* strains were allowed to develop from light spore inocula on nutrient agar, and one colony of each strain tested with α phage. Signs of disintegration of the colony were evident with each strain within 3 hr. of application of the phage.

Tests with other species of the genus Bacillus. The α-phage caused no damage to filaments or microcolonies of one strain each of *Bacillus cereus*, *B. mycoides*, *B. licheniformis* or *B. subtilis*, when these were tested in parallel with strains of *B. anthracis*. Plate 1, fig. 5, shows a microcolony of *B. cereus* after 4 hr. incubation on nutrient agar. Plate 1, fig. 6, shows a larger microcolony of *B. cereus* which had been treated with α-phage after 4 hr. incubation and then incubated for a further 1½ hr.

Other control measures. When α-phage was applied to a filament or microcolony, a similar filament or microcolony on the same plate was treated with a loop of sterile broth in the same way. Such control filaments always grew normally into microcolonies which never showed any fragmentation of their constituent filaments such as might have been produced by mechanical means.

Total time for identification of Bacillus anthracis *by the phage-lysis micro-test.* The time elapsing between inoculation of agar with a light spore suspension and the observation of convincing lysis of the resulting microcolonies was 5–7 hr. The shorter times were observed on medium enriched with PSB, where growth and lysis were slightly faster than on plain nutrient agar.

DISCUSSION

The specificity of the α-phage was established by McCloy (1951), who examined 171 strains of *Bacillus anthracis* and 244 other strains of other species of the genus *Bacillus*, by spotting a drop of the undiluted phage on a quarter plate spread previously with the bacterial strain to be tested. All the strains of *B. anthracis* were attacked by the phage, and only 2 of the 244 strains of other species were susceptible. These two were both *B. cereus* strains; another 54 strains of *B. cereus* were not attacked by the phage. Brown & Cherry (1955) found that α phage lysed all of 41 naturally occurring rough strains of *B. anthracis*, 2 of 89 strains of *B. cereus*, and none of 134 strains of other *Bacillus* species.

The evidence presented in this paper indicates that it should be possible to identify *Bacillus anthracis* by means of specific bacteriophage within 7 hr. of sowing an agar plate with suspected material.

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EXPLANATION OF PLATE

Figs. 1–6. Transmitted light 16 mm. objective. Magnification, ×150.

Fig. 1. Two filaments of *Bacillus anthracis*, approximately at right angles to each other, on nutrient agar inoculated with spores and incubated 4 hr. at 37°.

Fig. 2. Microcolony of *B. anthracis* on nutrient agar. 6 hr. at 37°.

Fig. 3. Filament of *B. anthracis* on nutrient agar, 4 hr. at 37°, $+1\frac{1}{4}$ hr. in presence of α-phage. Lysis in the right-hand half of the filament, apart from one very short segment. The original position of the lysed filament is shown by a groove in the agar.

Fig. 4. Same filament as in fig. 3, $\frac{1}{4}$ hr. later. Lysis at four additional points.

Fig. 5. Microcolony of *B. cereus* on nutrient agar inoculated with spores and incubated 4 hr. at 37°.

Fig. 6. Microcolony of *B. cereus* on nutrient agar, 4 hr. at 37°, $+1\frac{1}{2}$ hr. in presence of α phage. Normal growth appearances; no lysis.

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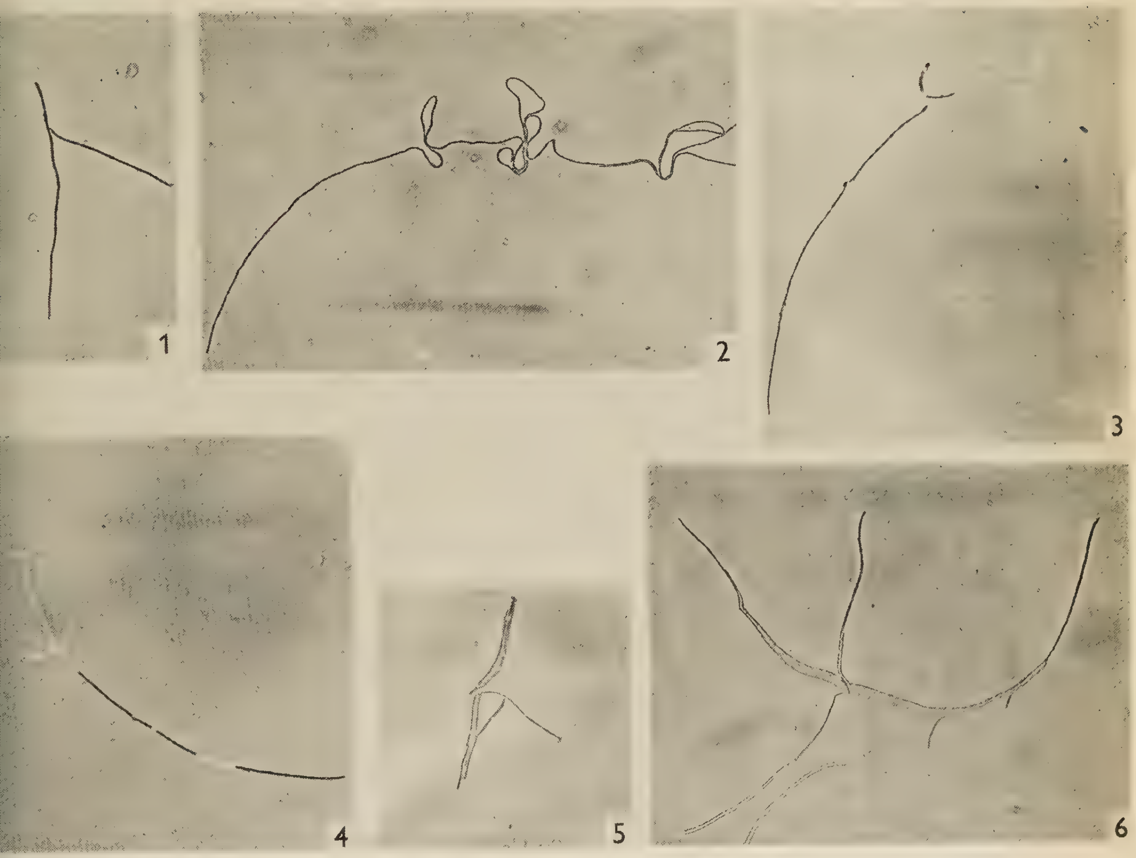

P. Chadwick—Rapid identification of *B. anthracis*. Plate 1

(*Facing p.* 634)

Boyd, J. S. K. & Bidwell, D. E. (1959). *J. gen. Microbiol.* 21, 635–651

The Q1 (A) Strains of *Salmonella typhimurium*: Induction Phenomena

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SUMMARY: Members of the Q1 (A) group of lysogenic strains of *Salmonella typhimurium* have a common bacterial component and differ only in their prophage content. All are inducible by ultraviolet (u.v.) radiation but, in the cultural conditions which were standard in the experiments here described, never more than 40% of the bacteria were induced, and this only with exposures which killed approximately 75% of the host strain, Q1. The latent period between irradiation and phage liberation was approximately 45 min. The number of particles per burst varied widely. The most striking difference in the reaction to irradiation of cultures of the different strains was that, without significant variation in the amount of free phage produced, some showed clearing while others did not. Clearing was not directly related to the percentage of bacteria induced, nor was it attributable to the production of a lysin by some strains and not by others. Clearing occurred in strains in which induction took place in bacteria which would otherwise have survived irradiation, and would have enlarged into filamentous forms. In strains in which no clearing occurred, induction took place in bacteria which had received a lethal dose of u.v. radiation, and not in the more lightly irradiated bacteria which were therefore left free to enlarge and so mask the lysis of the induced bacteria. Thus there is evidence that the induction of these closely related prophages is 'triggered off' by different stimuli directly related to the degree of damage to the host bacterium caused by irradiation.

Lwoff, Siminovitch & Kjeldgaard (1950) found that when lysogenic *Bacillus megaterium* was exposed to ultraviolet (u.v.) radiation under certain defined conditions it was 'induced': the innocuous prophage assumed virulent characters and multiplied at the expense of the host bacterium, producing a brood of free phage particles. Subsequent work has shown that many lysogenic bacteria of different species can be induced in this way, both by u.v. radiation and by other mutagenic agents. There is, however, considerable variation in the degree of induction produced in different bacteria and even in lysogenic bacteria of the same species (Gorrill & Gray, 1956).

Recently, a series of twelve lysogenic strains of *Salmonella typhimurium* was prepared by infecting an indicator strain, Q1, with the different type A phages which have been recovered from wild strains of that organism (Boyd & Bidwell, 1957). This will be referred to as the Q1 (A) series. In all members of the series the bacterial component is the same: only the prophages differ. Thus any variation in reaction which these lysogenic strains show must be attributed to the prophage. A few preliminary experiments showed that there was in fact a very considerable variation in response to u.v. radiation, and it was deemed of interest to explore this phenomenon more carefully. It must be re-emphasized that these phages are not artificially produced mutants of a common ancestor. They have all been recovered, in many cases repeatedly, from wild strains of *S. typhimurium* from cases of food poisoning or from carriers.

METHODS

Two indicator strains were used, *Salmonella typhimurium* Q1, which is sensitive to all the A phages, and *S. gallinarum* SL140, a streptomycin-resistant strain kindly provided by Dr B. Stocker. *S. gallinarum* is an efficient indicator for a wide range of Salmonella phages.

The culture media were those detailed previously (Boyd, 1950) and the buffer that of Visconti & Delbrück (1953). During incubation, cultures were neither aerated nor shaken, the active movements of the bacteria being deemed adequate to ensure collisions between them and the phage particles.

The optical density of the cultures from which growth curves were plotted was estimated by the EEL nephelometer; this instrument proved very suitable for the purpose, as readings could be made quickly and with little exposure of the culture tubes to light, so avoiding the risks of photorestoration.

For u.v. radiation a Philips Germicidal Lamp of 30 W. was used at a distance of 1 m. from the cultures, the lamp being allowed to heat up for 15 min. before being used. The efficacy of the irradiation was estimated by preliminary tests which will be described. This work has been in progress for some years. In earlier experiments there were certain unsuspected factors which affected the length of exposure. It has been possible to make accurate adjustments, and the corrected results of these earlier experiments have been embodied with those of later experiments. The exposures quoted throughout the paper are in terms of the later experiments.

To prepare bacteria for irradiation tubes of broth (10 ml.) were inoculated from overnight agar slopes, incubated for 3 hr. at 37°, and then centrifuged. The bacteria were resuspended in buffer of equal volume to the original cultures, and the contents of the tubes pooled. Two ml. of this was added to 8 ml. of broth for control purposes. Prepared in the above way, this control contains approximately 10^8 viable bacteria/ml. The remainder was placed in quantities of 3 ml. in 10 cm. Petri dishes, as many as required for the particular experiment. Each sample for irradiation was placed on a rocking platform at the appropriate distance from the lamp and the exposure was made by removing and replacing the lid of the Petri dish like the lens cap of a studio camera. After irradiation, 2 ml. of the suspension was added to 8 ml. of broth, giving the same concentration of bacteria as in the control. Incubation was carried out at 37° in a Grant water bath with a metal cover to exclude light.

The percentage of bacteria surviving exposure to u.v. radiation was estimated by making viable counts before and after irradiation. The percentage of bursts, i.e. of bacteria which undergo induction and lysis after irradiation, was found by diluting the irradiated culture to give approximately one organism/0·4 ml., and charging each of 100 sterile tubes (4 in. × $\frac{1}{2}$ in.) with this volume. To ensure that all induced bacteria burst, the tubes were incubated in the water bath for 2 hr. at 37°. Thereafter, to save unnecessary manipulation, a preliminary test was made by spotting a loopful from each tube on numbered sections of a plate of agar freshly sown with the indicator strain (a 'lawn'). After overnight incubation, the tubes in which bursts had occurred were identified by the presence of plaques in the numbered spots, and an accurate count of the phage particles in each of these tubes was made by standard methods to calculate the number of particles produced in each burst. As it is impossible to distribute the bacteria in the tubes with mathematical accuracy (i.e. when 100 bacteria are shared into 100 tubes with the theoretical distribution of 1 bacterium/tube, approximately one-third of the tubes are found to contain no bacteria, and certain of the remaining tubes 2, 3 and even 4 bacteria) a correction factor based on the Poisson distribution was used to convert 'tubes showing bursts' to 'estimated numbers of induced bacteria'. A few confirmatory experiments were carried out using a dilution estimated to give one bacterium/4 tubes: these gave comparable results.

No attempt was made, either by varying the medium or by other means, to increase the yield of phage particles in an irradiated culture.

Smears for microscopical examination were dried and fixed, either by methyl alcohol to be stained by Giemsa's stain, or by heat to be stained by borax methylene blue or carbol fuchsin. Special methods of staining designed to show internal structure were not employed.

RESULTS

Before proceeding to consider the effects of u.v. radiation on the Q1 (A) series, some recent observations on Q1, the parent strain, must be recorded. This strain was derived from a wild *Salmonella typhimurium*, carrying prophage A3, which was isolated from the faeces of a patient suffering from food poisoning. During a prophage substitution experiment a few bacteria in the cultures were 'cured' of their A phage infection, and a colony which grew from one of these cured organisms was the origin of the Q1 strain. At first, Q1 was thought to be phage-free, as no phage arising from it could be detected on the indicator strains 1404 and 1411 used at that time. However, Dr B. Stocker has recently found (personal communication) that Q1 contains a phage for which *S. gallinarum* strain SL 140 is an indicator—an observation which was readily confirmed. On a lawn of SL140, the phage from Q1 produces small punched-out plaques, about 0·1–0·15 mm. in diameter, which under high magnification are seen to have a minute central spot of bacterial growth. This phage is neutralized by the antiserum of phage B5 (Boyd, 1950), and further resembles B5 by showing considerable heat resistance: a limited number of particles survive exposure for 30 min. to 70°, but all are destroyed by heating for 30 min. at 75°. It differs from B5, of course, in its host range. This phage from Q1 has been designated B5d.

Phage lysates, prepared from the Q1 (A) series, were spotted on a lawn of *Salmonella gallinarum* strain SL140. This revealed in every case plaques of the A phage and of phage B 5d. All these strains are therefore doubly lysogenic, carrying both their particular A phage and B 5d.

When cultures of the Q 1 (A) series are induced by u.v. radiation, it is found that the A phage take priority over B 5d. The two phages are induced independently. On rare occasions both phages have been found in the same tube in the burst experiments to be detailed later, but no more often than could be accounted for by the occasional presence of two bacteria instead of one in the tube. The percentage of B 5d bursts is much lower in irradiated Q 1 (A) cultures than in irradiated Q 1, and for some reason, for which no explanation can be offered, the number of B 5d phage particles released per burst is also much lower in irradiated Q 1 (A) strains than in irradiated Q 1.

In the experiments to be recorded, the presence of phage B 5d in strain Q 1 has no particular significance, and to avoid confusion no reference will be made to it, but as a matter of interest, the B 5d figures are embodied in Fig. 10.

Irradiation of the parent strain Salmonella typhimurium *Q* 1

Morphological changes resulting from irradiation. Stained smears were prepared at intervals from an irradiated culture of *Salmonella typhimurium* Q 1 subsequently incubated at 37°. Immediately after irradiation and before incubation, numerous organisms were seen in various stages of division. After incubation for 15 min. dividing forms were fewer in number and after 30 min. they were scanty. After 45 min. many organisms were seen to be swollen and elongated, and a few, about twice the normal size, were boat-shaped, with deeply stained ends and a clear centre. The process of enlargement continued up to 90 min., when numerous filamentous bacteria measuring 11–20 μ were present. Subsequently, these filaments grew proportionately fewer in number, while small dividing forms reappeared. Some of the filaments stained irregularly, others were beaded, and some fragmented, giving a streptobacillus-like appearance. After overnight incubation of the irradiated broth cultures, occasional filaments were still to be seen and, in general, the bacteria showed much irregularity in size, the average being greater than that of the organisms in the original culture. The fate of the filamentous forms was followed by keeping a live preparation under continuous observation with phase-contrast illumination, using a technique previously described (Boyd, 1949). A minority were found to remain in this enlarged state indefinitely. The majority divided after 90 min. or more of incubation from the time of irradiation, and the daughters continued to divide. Those of the first generation were from 2 to 3 times the size of normal bacteria, those of the second generation were still enlarged, though not so big as their parents, and those of subsequent generations were approximately normal in size. Thus the majority of the filamentous forms were not moribund as might at first be supposed, but in time divided and produced apparently normal progeny. (A similar observation was made in the case of *Escherichia coli* by Deering & Setlov, 1957.) Only a minority of the bacteria which became enlarged and swollen failed to survive. From this it may be deduced that the majority of the bacteria which were killed by irradiation (apart from the action of induction) were affected to such an extent that they did not increase in size but were, as it were, killed outright.

Lethal effect of u.v. radiation. Strain Q1, after the standard preparatory treatment, was irradiated for varying periods, and the lethal effect of this treatment was estimated by counting the viable bacteria in samples taken before and after irradiation. The findings in repeated experiments of the kind were remarkably consistent, though it was noted that minor variations occurred which were related to the concentration of the bacteria in the suspension at the time of irradiation. Thus, with a denser suspension the percentage of surviving bacteria was higher, a result probably attributable to 'shadowing'. The results are shown in Table 1.

Table 1. *Lethal effect of ultra-violet radiation on* Salmonella typhimurium *strains Q*1, *Q*1 (*A*1*b*) *and Q*1 (*A*1*c*)

Suspension of *Salmonella typhimurium*, strains Q1, Q1 (A1b) and Q1 (A1c), were prepared for irradiation by the methods described. The viable bacteria were counted (1) in the suspensions immediately before irradiation, and (2) in portions of the suspensions immediately after exposure to u.v. radiation for the times indicated. The 'survivors' are shown as a percentage of the viable bacteria in the suspension before irradiation. The figures given for exposures of 5 and 10 sec. are the mean of several experiments.

Salmonella typhimurium strain	Length of exposure (in sec.) to u.v. radiation: 2½	5	10	20
	Percentage of bacteria which survive irradiation			
Q1	72	51 ± 2	25 ± 2	8
Q1 (A1b)	50	28 ± 2	5 ± 0·2	1
Q1 (A1c)	69	41 ± 5	23 ± 3	7

Effect of u.v. radiation on turbidity curves. The process of growth with arrested division is reflected in the turbidity curves plotted in Fig. 1. The preliminary increase in opacity, which occurred in the first 90 min. of incubation, resulted from the enlargement of the bacteria and not, as in the control, from multiplication. The flattening of the curve which is seen for the next 30–60 min. cannot be attributed to lysis resulting from the induction of phage B5d, for this lysis, as shown by the rising titre of free phage, occurred between 45 and 60 min. after irradiation, at which time the turbidity curve was still rising: it is evidence of the persisting effect of irradiation on the metabolism of the surviving bacteria, causing a slowing down of their growth rate.

It is noteworthy that the more lightly irradiated cultures showed a greater increase in opacity during the first 90 min. of incubation than did those which were more heavily irradiated, a fact which suggests that minor damage interfered with division without greatly influencing growth, whereas more severe damage brought both division and growth to an end by killing the organism.

Ultraviolet radiation of free particles of type A phages

Latarjet, Morenne & Berger (1953) have shown that coliphage T_2 is quickly destroyed by irradiation. Both temperate and virulent A phages are much more resistant. The survival rates of coliphage T_2 and Salmonella phage A1b are compared in Fig. 2. The other phages of the Q1(A) series are similarly resistant, and the exposure of the lysogenic cultures to irradiation for 20 sec. (the longest time adopted in most of the experiments which follow) produced only a negligible decrease in the number of free phage particles when counts were made immediately after exposure. It is of interest to recall that these same phages are very resistant to heat, and survive exposure to a temperature of 80° for 30 min.

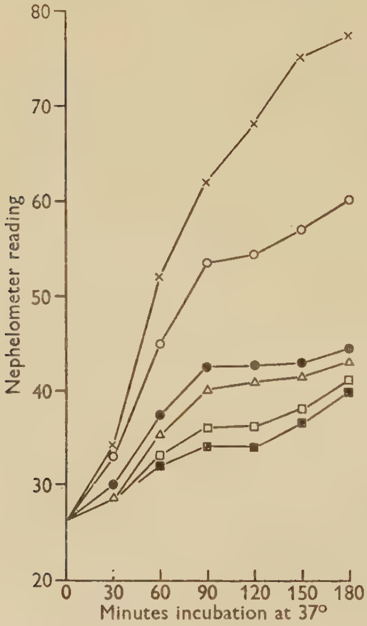

Fig. 1

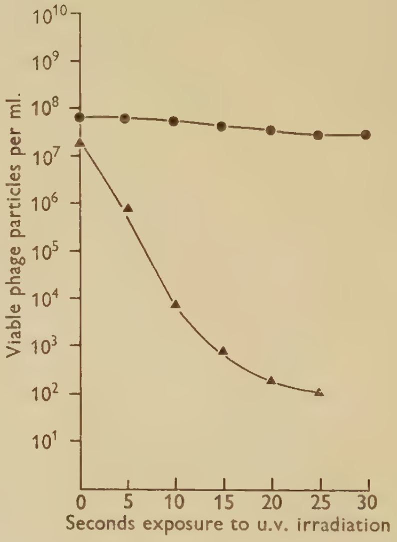

Fig. 2

Fig. 1. Turbidity curves of incubated cultures of *Salmonella typhimurium*, strain Q1, after varying exposures to u.v. radiation. With increasing exposures the curves become lower and flatter, but show no abrupt fall indicating mass lysis. Seconds irradiation: ×, 0; ○, 2·50; ●, 5·00; △, 9·5; □, 10·0; ■, 12·5.

Fig. 2. Lethal effect of u.v. radiation on free phage particles of coliphage T_2 and Salmonella phage A1b. ▲, Bacteriophage T_2; ●, bacteriophage A1b.

Ultraviolet radiation of the Q1(A) series of Salmonella phages

Preliminary tests. A series of preliminary observations was made by irradiating all 12 lysogenic strains for 5 sec. and thereafter incubating and recording turbidity at intervals for 3 hr. The resultant curves varied considerably. In two there was evidence of well-marked lysis, i.e. almost complete clearing of the culture; in others there was less obvious lysis, while in the remainder there was retardation of growth of varying degrees with no fall in the curve to suggest mass lysis.

The 12 strains were then irradiated for 10 sec., and the titre of free phage particles estimated after incubation for 90 min. The results, together with an assessment of the degree of clearing which occurs, are shown in Table 2. It will be seen that while all 12 strains were induced, there was some variation in the final concentration of phage. This however bore no constant relationship to the degree of clearing. The highest titre was found in the culture of Q1 (A2d), which showed well-marked clearing, but other types which showed no clearing, such as Q1 (A1c), had almost as high a titre as Q1 (A1b), in which clearing was marked.

Table 2. *Comparison of results of irradiation of the Q1 (A) series of* Salmonella typhimurium

Bacterial lysis was estimated by nephelometer readings of broth cultures taken at intervals during incubation at 37°. ++ denotes the degree of clearing seen in Fig. 3; − denotes absence of clearing as seen in Figs. 1 and 4; + and ± denote intermediate degrees. Suspensions were prepared for irradiation by the methods described, and the free phage particles in these suspensions were counted before exposure to u.v. radiation. Portions of each suspension were irradiated for 5 sec., and thereafter incubated together with unirradiated suspensions as controls. The free phage particles were counted after incubating for 90 min. The control shows the normal increase in free phage which is found in an actively growing lysogenic culture. The much higher count in the irradiated sample results from induction of a proportion of the lysogenic bacteria.

	Degree of bacterial lysis	Phage count before irradiation	Phage count in control culture incubated 90 min.	Phage count in irradiated culture incubated 90 min.
Q1 (A1a)	+	$5{\cdot}2\times10^{4}$	$1{\cdot}8\times10^{6}$	$9{\cdot}0\times10^{8}$
(A1b)	++	$2{\cdot}4\times10^{5}$	$1{\cdot}4\times10^{6}$	$1{\cdot}9\times10^{9}$
(A1c)	−	$2{\cdot}7\times10^{4}$	$8{\cdot}0\times10^{5}$	$1{\cdot}6\times10^{9}$
(A1d)	−	$2{\cdot}3\times10^{4}$	$5{\cdot}6\times10^{5}$	$2{\cdot}7\times10^{9}$
(A2a)	+	$1{\cdot}8\times10^{5}$	$1{\cdot}0\times10^{6}$	$3{\cdot}8\times10^{9}$
(A2b)	±	$7{\cdot}2\times10^{3}$	$1{\cdot}3\times10^{5}$	$2{\cdot}3\times10^{9}$
(A2c)	+	$1{\cdot}7\times10^{3}$	$6{\cdot}8\times10^{5}$	$1{\cdot}7\times10^{8}$
(A2d)	++	$3{\cdot}9\times10^{5}$	$1{\cdot}0\times10^{6}$	$5{\cdot}2\times10^{9}$
(A2e)	+	$3{\cdot}6\times10^{3}$	$6{\cdot}0\times10^{4}$	$2{\cdot}7\times10^{8}$
(A2f)	−	$4{\cdot}8\times10^{3}$	$7{\cdot}2\times10^{4}$	$1{\cdot}1\times10^{8}$
(A3)	−	$4{\cdot}8\times10^{5}$	$1{\cdot}1\times10^{7}$	$1{\cdot}4\times10^{9}$
(A4)	−	$1{\cdot}2\times10^{5}$	$2{\cdot}8\times10^{6}$	$6{\cdot}4\times10^{8}$

Selection of strains Q1 (A1b) and Q1 (A1c) for detailed investigation. Two strains were selected for detailed investigation, namely Q1 (A1b) which showed well-marked clearing, and Q1 (A1c), which showed retardation of growth but no clearing. A third strain, Q1 (A1a), which showed a limited degree of clearing, was also examined in the same detail; the results of the various investigations carried out on this strain lay so consistently in an intermediate position between those of the other two, that nothing is to be gained by recording them.

Prophages A1b and A1c confer a high degree of immunity on their host bacteria. Not only do strains Q1 (A1b) and Q1 (A1c) show mutual cross-immunity to the phages they produce, but they are also, with minor exceptions, resistant to all the A phages (Boyd & Bidwell, 1957). Conversely, phages A1b and A1c multiply freely on 6 of the 12 strains of the Q1 (A) series. There are, however, some minor differences in the cross-immunity reactions of the two strains by which they can be identified; they also show variations in plaque characters and in host range. Nevertheless, they appear to be more closely related than any other members of the A series, and it is therefore the more interesting that they should differ so widely in their reactions to u.v. radiation. Prophage A1b is the phage of Lilleengen's LT22 strain 409 (Lilleengen, 1948) used by Zinder & Lederberg (1952) in their transduction experiments.

Morphological changes after irradiation. On incubation at 37°, the morphological characters of the bacteria in irradiated cultures of these lysogenic strains differ notably. Strain Q1 (A1c), which has growth curves resembling those of strain Q1, but slightly flatter, undergoes changes very similar to those shown by Q1, which have already been described. Division is arrested, and many of the organisms become grossly enlarged and filamentous. After incubation for 90 min. these enlarged forms become progressively fewer, but some are still present in an overnight culture.

Strain Q1 (A1b), which shows well-marked clearing, presents a different picture. In the first 45 min. of incubation it develops in much the same way as Q1, except that a larger percentage of the organisms have a boat-like appearance, with polar staining. From this stage onwards, progress is very different. The number of bacteria in the smear notably decreases; only a few filamentous forms, so conspicuous a feature in corresponding smears of Q1 and Q1 (A1c), develop; the boat-like organisms disappear, and most of the remaining bacteria are of normal size.

Turbidity curves. Cultures of strains Q1 (A1b) and Q1 (A1c) were irradiated and incubated. The turbidity of the cultures was recorded at 30 min. intervals, and the curves plotted (Figs. 3, 4). These conform to the findings of the preliminary experiments. In Q1 (A1b) well-marked clearing occurred after incubation for about 90 min. The general pattern of the curve was not changed by increased exposure, the only significant difference being that the cultures which received the longer exposures had lower turbidity readings. The curves of Q1 (A1c) are much like those of Q1 (Fig. 1), but run at a lower level. No noticeable clearing occurred, though the curves were flattened.

Counts of viable bacteria in cultures immediately after irradiation. The lethal action of u.v. radiation on strains Q1, Q1 (A1b) and Q1 (A1c) is shown in Table 1. Q1 (A1c) showed a slightly lower survival ratio than Q1; in Q1 (A1b) the survivors were markedly fewer than in the first two.

Counts of viable bacteria in irradiated cultures after different periods of incubation. It seemed possible that the clearing which occurred in an irradiated culture of strain Q1 (A1b) might have resulted from the action of a lysin liberated from the induced bacteria. To test this hypothesis, cultures of Q1 (A1c) and Q1 (A1b), prepared in the standard way, were irradiated for 5 sec. and incubated in parallel with an unirradiated control. Counts of the viable bacteria in both preparations were made immediately after irradiation. It was assumed that the lysin was unlikely to function unless it was present in a concentrated form. Accordingly, viable counts were made at intervals (1) from the original irradiated culture containing approximately 10^8 bacteria (living or dead)/ml., and (2) from secondary cultures prepared by diluting the original $0{\cdot}2 \times 10^{-5}$, giving a content of 2×10^2 bacteria (living or dead)/ml., and a correspondingly decreased concentration of any lysin which might be present. The results are shown in Figs. 5 and 6. The turbidity curves of controls and irradiated cultures follow the usual pattern. The viable bacteria are plotted as a percentage of the number originally present in the unirradiated control. In both irradiated cultures the number of viable bacteria decreased during the first 60 min. of incubation, but fairly rapid multiplication (which, as would be expected, was more marked in the diluted cultures containing fresh medium) set in after about 90 min. The decrease in viable bacteria was greater in the undiluted cultures, indicating the presence of some lethal agent. But the difference between strain Q1 (A1b), in which clearing occurred, and strain Q1 (A1c) in which there was no clearing, was not sufficient to be regarded as significant.

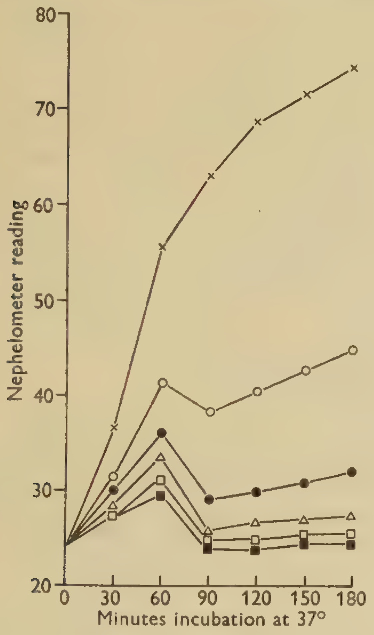

Fig. 3

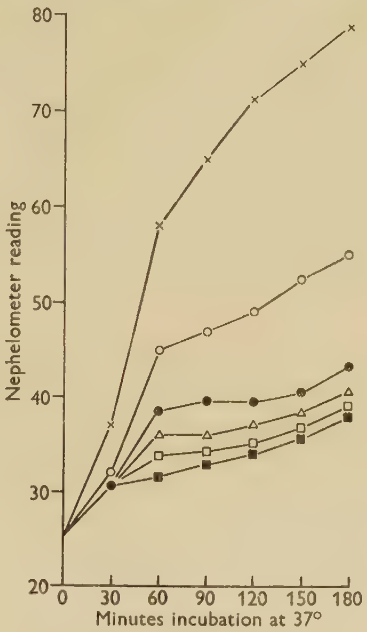

Fig. 4

Fig. 3. Turbidity curves of incubated cultures of *S. typhimurium*, strain Q1 (A1b), after varying exposures to u.v. radiation. After incubation for 60 min. clearing occurs at all exposures. Seconds irradiation: ×, 0; ○, 2·5, ●, 5; △, 7·5; □, 10; ■, 12·5.

Fig. 4. Turbidity curves of incubated cultures of *S. typhimurium* strain Q1 (A1c) after varying exposures to u.v. radiation. The curves are similar to those of strain Q1 (Fig. 1), but run at a lower level. Seconds irradiation: ×, 0; ○, 2·5; ●, 5; △, 7·5; □, 10; ■, 12·5.

Liberation of endolysin. Experiments were carried out along the lines suggested by Jacob & Fuerst (1958). Cultures of strains Q1 (A1b) and Q1 (A1c), prepared by standard methods, were irradiated for 5 sec., and different samples were incubated for 50 min. (the point at which clearing was about to begin in Q1 (A1b)) and 120 min (when clearing was complete). These samples, both with and without the addition of Tween 80 (to counteract possible adsorption), were centrifuged and filtered through Gradocol membranes to remove the phage, and were then tested against cultures of strains Q1, Q1 (A1b) and Q1 (A1c) which had first been treated with ethylenediamine tetra-acetic acid. There was no detectable clearing, from which it would appear that no endolysin was liberated in the irradiated cultures.

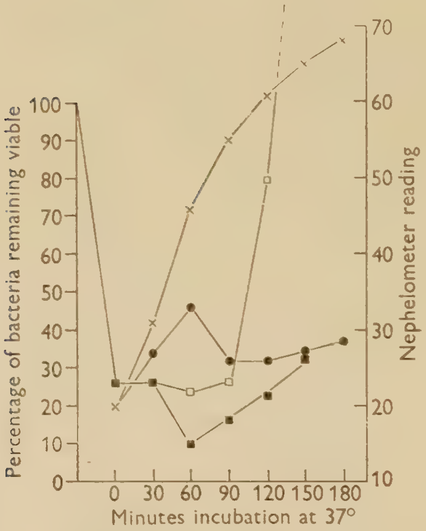

Fig. 5

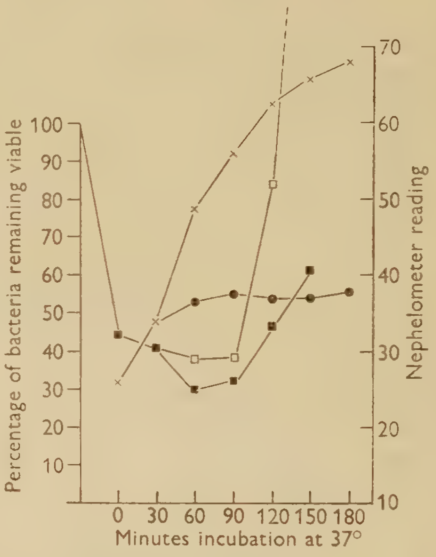

Fig. 6

Fig. 5. Progressive lethal action of u.v. radiation on *S. typhimurium* strain Q1 (A1b). In an undiluted culture the count of viable bacteria falls during the first 60 min. of incubation. A portion of the same culture diluted $0{\cdot}2 \times 10^5$ shows no such fall. ×, Turbidity curve of control culture; ●, turbidity curve of irradiated culture; □, count of viable bacteria made from diluted culture; ■, count of viable bacteria made from undiluted culture.

Fig. 6. Progressive lethal action of u.v. radiation on *S. typhimurium* strain Q1 (A1c). The fall in the viable count is slightly less than in strain Q1 (A1b) (Fig. 5). ×, Turbidity curve of control culture; ●, turbidity curve of irradiated culture; □, count of viable bacteria made from diluted culture; ■, count of viable bacteria made from undiluted culture.

Influence of length of exposure on induction as evidenced by the liberation of free phage. To ascertain the effect of different lengths of exposure to u.v. radiation, a series of standard suspensions of the two lysogenic strains was irradiated for different periods, incubated for 90 min. (the time at which the optimum phage titre was reached in an induced culture), diluted in streptomycin broth to kill the surviving bacteria, and counted on *Salmonella gallinarum*, strain SL140. The results are plotted in Fig. 7. The maximum titre was reached after 5–10 sec. exposure. The number of particles liberated by the induction of strain Q1 (A1b) was higher than in the corresponding preparation of Q1 (A1c), but not strikingly so. The titre of free phage has varied slightly in different experiments of this type, probably in relation to the bacterial count of the suspension, but the pattern of the opacity curve has been constant. On no occasion has Q1 (A1c) shown detectable clearing.

Length of exposure and percentage of bacteria induced. Suspensions of strains Q1 (A1b) and Q1 (A1c), prepared by the standard technique, were irradiated for different lengths of time, and thereafter the number of bursts was determined by the method already described. In some cases repeated experiments were performed; the results shown in Table 3 are the mean of the findings. An exposure of 5 sec. gave the highest number of bursts. With exposures of $2\frac{1}{2}$ sec. the number of bacteria induced in cultures of strain Q1 (A1b) was slightly higher than in the case of Q1 (A1c). With an exposure of 5 sec. induction was practically alike in both. With an exposure of 10 sec., the induction of Q1 (A1c) was much decreased, being only about half of that of Q1 (A1b). As an exposure of 5 sec. produced a well-marked clearing in subsequently incubated cultures of strain Q1 (A1b), and none in Q1 (A1c) (see Figs. 3, 4) it can be concluded that clearing is not to be explained solely in terms of the percentage of bacteria in a culture which are induced.

Table 3. *Influence of length of exposure to ultra-violet radiation on the percentage of bacteria induced*

Standard suspensions of bacteria were irradiated for the times shown. The suspensions were then diluted to give an average of 1 bacterium/0·4 ml. and distributed in 0·4 ml. quantities in 100 small test tubes. In some experiments the dilution was greater, giving an average of 1 bacterium/1·6 ml.: this was also distributed in 0·4 ml. quantities in 200 small test tubes, giving an average of 1 bacterium/4 tubes. The tubes were incubated for 2 hr. and thereafter tested for the presence of free phage particles (indicating that the bacterium had been induced). A correction factor based on the Poisson distribution was used to convert 'tubes in which bursts occurred' to 'percentage of bacteria induced'; this is shown as the nearest whole number. Several experiments were carried out with exposures of 5 and 10 sec.

Salmonella typhimurium strain	Length of exposure (in sec.) to u.v. radiation			
	$2\frac{1}{2}$	5	10	20
	Percentage of bacteria which 'burst' as a result of induction of the A prophage			
Q1 (A1b)	29	36±5	35±3	24
Q1 (A1c)	25	36±2	16±2	5·0

Number of phage particles produced by an induced bacterium. There was gross variation in the burst size, i.e. the number of phage particles produced by different bacteria when induced. The results of several experiments with *Salmonella typhimurium* are compiled in Table 4. In many cases the high figures at the upper end of the range are to be attributed to the presence of more than one induced bacterium/tube, but even when this is taken into account the spread is very wide. There has also been considerable variation in different experiments: for no obvious reason the burst size was much above the average

Table 4. *Range of 'burst' sizes*

These counts were made on the 'bursts' recorded in Table 3. The striking feature is the wide scatter of burst sizes in every experiment, including those experiments in which the dilution was such that the chances of two induced bacteria being present in the same tube were remote.

Salmonella typhimurium strain	Exposure to u.v. radiation (sec.)	Viable bacteria per ml. before irradiation	Theoretical distribution of bacteria per tube	No. of tubes showing bursts	Lowest no. of particles	Highest no. of particles
Q1 (A1c)	5	$1\cdot0 \times 10^8$	1/1	30/100	18	420
	5	$1\cdot2 \times 10^8$	1·2/1	38/100	26	624
	5	$1\cdot14 \times 10^8$	0·285/1	19/200	48	392
	5	$1\cdot08 \times 10^8$	0·27/1	21/200	54	384
	10	$1\cdot1 \times 10^8$	1·1/1	13/100	82	1308
	10	$1\cdot03 \times 10^8$	1·03/1	17/100	38	536
	10	$0\cdot8 \times 10^8$	0·8/1	12/100	80	598
	10	$1\cdot07 \times 10^8$	1·07/1	15/100	12	470
Q1 (A1b)	5	$1\cdot16 \times 10^8$	1·16/1	38/100	6	416
	5	$0\cdot9 \times 10^8$	0·9/1	33/100	8	394
	5	$1\cdot1 \times 10^8$	0·275/1	17/200	22	322
	5	$1\cdot08 \times 10^8$	0·27/1	16/200	16	232
	10	$0\cdot84 \times 10^8$	0·84/1	23/100	8	600
	10	$0\cdot9 \times 10^8$	0·9/1	29/100	32	300
	10	$1\cdot04 \times 10^8$	0·04/1	32/100	12	398

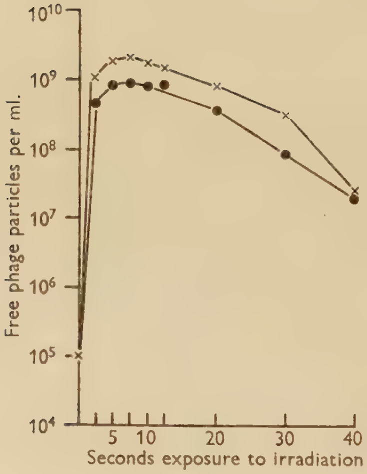

Fig. 7

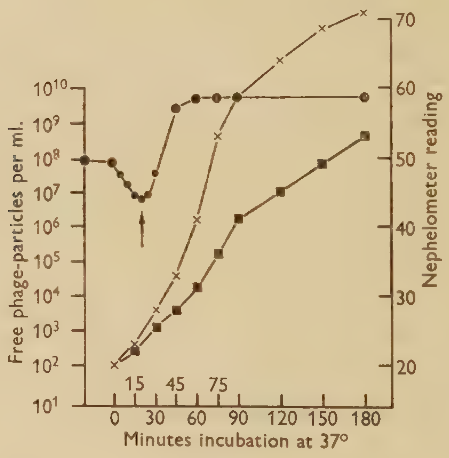

Fig. 8

Fig. 7. The titre of free phage particles, resulting from the induction of *S. typhimurium* strains Q1 (A1b) and Q1 (A1c), in cultures exposed to ultraviolet radiation for varying periods. Optimum induction results from exposures of 5–10 sec. Strain Q1 (A1b) gives a higher titre than Q1 (A1c). ×, Phage A1b; ●, phage A1c.

Fig. 8. Adsorption of phage A1b to *S. typhimurium*, strain Q1, with subsequent multiplication. The arrow indicates the point at which phage production begins—approximately 20 min. ×, Turbidity curve of Q1; ■, turbidity curve of Q1+phage A1b; ●, titre of phage A1b.

of the others in three experiments out of fifteen. This is in contrast to the other figures set out in this paper, such as the percentage of survivors and the percentage of induced bacteria, where the findings in different experiments have been relatively constant.

Latent period and phage production curve. When an actively growing culture of strain Q1 containing approximately 10^8 bacteria/ml. was exposed to temperate phage A1b at a concentration calculated to give a phage: bacterium ratio of 1:1, the bacteria quickly adsorbed the phage particles. Production of new phage began in about 20 min. and ended in about 90 min. (Fig. 8). Lysogenic strain Q1 (A1b), prepared as for irradiation but incubated without irradiation, showed a steady increase in free phage which reached its maximum in *c.* 45 min., and thereafter settled down to run at a more or less constant value for the rest of the period of incubation (Fig. 9). Irradiated strain

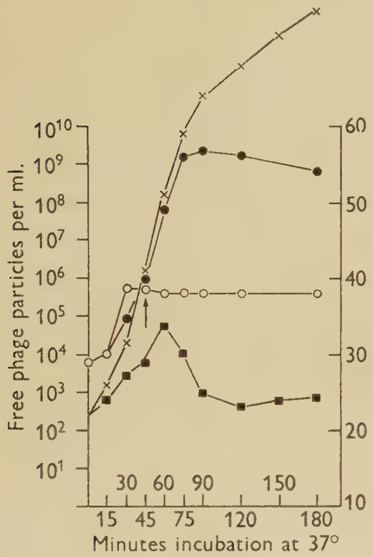

Fig. 9

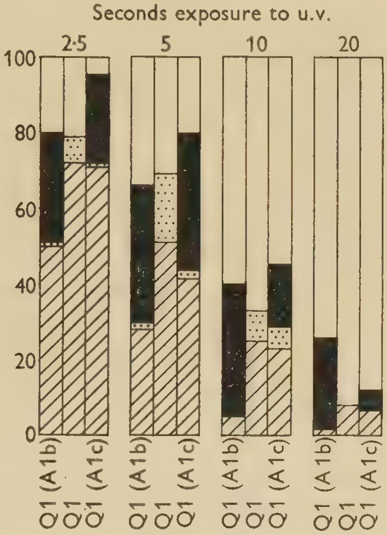

Fig. 10

Fig. 9. Latent period between irradiation of *S. typhimurium* strain Q1 (A1b) and the liberation (as a result of induction) of free phage particles. The arrow (at 45 min.) indicates the point at which the curve of phage production in the irradiated culture breaks away from the curve of 'normal' phage production in the control. ×, Turbidity curve of Q1 (A1b); ■, turbidity curve of Q1 (A1b) irradiated 5 sec.; ○, titre of free phage in non-irradiated Q1 (A1b); ●, titre of free phage in irradiated Q1 (A1b).

Fig. 10. Relationship of induction to the degree of damage which u.v. radiation produces in the host bacterium. The central column in each section serves as a control, and shows the percentage of bacteria in cultures of *S. typhimurium*, strain Q1 (non-lysogenic with A phage), which survive irradiation. At all degrees of exposure, induction in strain Q1 (A1b)—shown in the columns to the left of the control—occurs partly in bacteria which, in terms of the control, strain Q1, would have survived irradiation, and partly in bacteria which would not have survived. In strain Q1 (A1c) (to the right of the control) the bacteria which are induced are mostly those which would not have survived irradiation. As a matter of interest, induction due to phage B5d is also shown in this diagram. ▨, Viable bacteria; ▦, bacteria destroyed by the induction of phage B5d; ■, bacteria destroyed by the induction of phage A1b or A1c; □, bacteria destroyed by u.v. irradiation.

Q1 (A1b) produced free phage at the same rate as the non-irradiated control for the first 45 min., during which time the curves ran parallel. Thereafter the curves diverged, for the titre of free phage particles in the irradiated culture became rapidly higher between the 45th and 90th min., after which it fell slightly as a result of adsorption of the phage particles to the multiplying lysogenic bacteria, which had survived irradiation.

This experiment was carried out with several of the Q1 (A) strains with similar results. Thus, in these lysogenic strains of *Salmonella typhimurium*, the latent period between irradiation and phage production, when incubation is carried out in a water bath, is 45 min. This exceeds by 25-30 min. the latent period between the exposure of strain Q1 to temperate A phage and the beginning of phage liberation.

DISCUSSION

All 12 members of the Q1 (A) series of *Salmonella typhimurium* strains, which differ from each other only in their prophage content, can be induced by u.v. radiation, but the percentage of bacteria in which induction occurs is relatively low. With the technique used it rarely exceeded 40 %, and in many cases was much below this figure. The pattern of the reaction in each lysogenic organism is constant and repeatable. The degree of induction cannot be increased beyond a certain point by lengthening the exposure to u.v. radiation. By the methods described, the maximum liberation of free phage particles is achieved by an exposure of from 5 to 10 sec. It is noteworthy that 50–75 % of the bacteria in cultures of the parent strain Q1 are killed by such exposures, and many of the survivors are considerably 'damaged'. The low rate of induction in the Q1 (A) series differs from that observed by Lwoff (1953) in *Bacillus megaterium* where, with the technique Lwoff used, 'almost the entire bacterial population was affected'. The wide variation in burst size is comparable to that observed in other systems (Delbrück, 1945).

The prolonged latent period is similar to that noted in induced *Bacillus magaterium* by Lwoff *et al.* (1950) who found that the irradiated bacterium divided twice before productive phage development started. There is, however, no suggestion that the Q1 (A) series divide after irradiation. This is clearly demonstrated in Q1 (A1b), where induction occurs mainly in those bacteria which otherwise—in the absence of induction—would have grown, without division, into giant filaments. Nevertheless, as the normal division time of strain Q1, in this particular cultural environment, is about 30 min. and the normal development time of phage A1b is 15–20 min. (Fig. 8), it seems possible that the liberation of the prophage which ushers in productive development takes place during some disturbance about the time when the nucleus would normally divide.

The main interest of this work centres round the fact that, in certain members of the series, induction is accompanied by clearing of the culture, while in others it is not, a phenomenon well seen in the two strains selected, Q1 (A1b) and Q1 (A1c). What causes this difference? The first and most obvious possibility which suggests itself is that clearing may be related to the percentages of bacteria in the culture which are induced. While this must play a part, it is not the only factor involved, for, as can be seen from Table 3, the percentage of bacteria induced in these two types by $2\frac{1}{2}$ and 5 sec. exposure to u.v. radiation is not significantly different, while the percentage of Q1(A1c) bacteria induced by 5 sec. exposure is higher than the percentage of Q1 (A1b) exposed for 20 sec., yet strain Q1 (A1b) shows well-marked clearing at all these exposures, while Q1 (A1c) does not (Figs. 3, 4).

Conceivably, clearing might be due to something in the nature of 'lysis-from-without' (Andrewes & Elford, 1932) brought about by the action of a high concentration of phage particles. Before the deoxyribonucleic acid of a phage particle enters the cytoplasm of a bacterium to which it has become adsorbed, the wall of the bacterium is breached by an enzyme present in the tail of the phage (Puck & Lee, 1954). When a large number of 'holes' of this kind are made at the same time, rupture of the bacterium due to weakening of the cell wall may occur. Lysogenic bacteria do in fact adsorb their homologous phage, but in experiments (unpublished) we have been unable to produce lysis-from-without by exposing lysogenic bacteria to high concentrations of homologous phage. Further, even if such a reaction did occur, there is no reason why it should operate in the case of strain Q1 (A1b) and not in Q1 (A1c), as adsorption is alike in both strains.

Again, it is possible that 'endolysin' might be liberated from irradiated strain Q1 (A1b) and not from Q1 (A1c), but in fact no endolysin could be detected. While the number of viable bacteria decreased during the first 45 min. of incubation of irradiated cultures (Figs. 5, 6) there was no marked difference between the percentage of bacteria killed in strains Q1 (A1b) and Q1 (A1c). Thus there is no evidence to link the clearing in strain Q1 (A1b) with the action of a lysin.

By taking the percentage of survivors in an irradiated culture of strain Q1 as a control, and comparing with this figure the percentage of survivors and the percentage of 'bursts' in irradiated strains Q1 (A1b) and Q1 (A1c), certain significant facts emerge. This comparison is made graphically in Fig. 10. In strain Q1 (A1b), induction occurs partly in bacteria which would otherwise have survived the effects of irradiation, and partly in bacteria which would not have survived irradiation, more in the former than in the latter. In Q1 (A1c), the majority of the inductions are in bacteria which would not have survived irradiation, so that the remaining viable bacteria are almost as numerous as in the control, Q1.

It might be thought that this destruction of viable bacteria, leaving only a few to multiply and make good those lost by induction, might bring about clearing. Here again a comparison of other experimental findings shows that, whatever part this may play, it is not the sole cause. Strain Q1 (A1b) when irradiated for 5 sec. has a higher percentage of survivors than Q1 (A1c) irradiated for 10 sec. (Table 1); the former shows well-marked clearing, the latter shows no clearing (Figs. 3, 4). Nevertheless, the clearing is in part related to the particular bacteria in which induction occurs. Microscopic examination of smears made from incubated irradiated cultures of strains Q1 and Q1 (A1c) show that these are alike in that a considerable percentage of the bacteria increase in size without dividing for approximately 90 min., becoming up to 6 times their normal size. Similar smears from Q1 (A1b) reveal only small numbers of enlarged forms, many of which disappear at an early stage, no doubt as the result of induction. From these microscopical findings it may be concluded that induction in strain Q1 (A1c) occurs in the heavily damaged bacteria in which both growth and division are arrested, whereas in Q1 (A1b) induction occurs in the more lightly damaged organisms which, in the absence of induction, would have enlarged and ultimately multiplied. This conclusion dovetails neatly into the findings presented in Fig. 10. Thus the clearing in strain Q1 (A1b) results partly from the induction of a proportion of the non-viable bacteria killed by irradiation, but mainly from the induction of a large proportion of the remaining viable bacteria which disintegrate before they can enlarge. On the other hand, irradiated Q1 (A1c) fails to clear because the lysis of the non-viable, non-enlarging bacteria is masked by the gross increase in size of the surviving bacteria.

The validity of these conclusions was tested by examining Q1 (A2d), a strain which when irradiated clears well, though not quite so well as Q1 (A1b). It was found that induction occurred partly in lightly affected bacteria and partly in organisms which had received a fatal dose of u.v. radiation. Enlarged forms were to be seen in smears, but these were less numerous than in corresponding smears of Q1 (A1c). On the other hand, the percentage of bacteria induced was higher than in Q1 (A1b) and Q1 (A1c).

It can therefore be concluded that clearing depends mainly on two factors. The first and most important is the level at which induction takes place in relation to the lethal action of the u.v. radiation on the bacteria. Clearing is most marked when induction occurs in lightly damaged organisms which in the absence of induction would enlarge and ultimately divide. It becomes less obvious where the induced bacteria are those which have received a lethal dose and consequently do not enlarge. The second and less important factor, as far as these particular strains are concerned, is the percentage of bacteria induced, though this must obviously play a part.

The fact that induction occurs at different degrees of irradiation damage is of considerable general interest. The most plausible explanation of induction, and one that commands general acceptance, is that the prophage, normally attached to the chromosome, and in this condition inactive and innocuous, is freed from its attachment by processes initiated by u.v. radiation, and assumes a productive or virulent role. If this theory is valid, it would appear that prophage A1b is activated by a hit which is less lethal to the host bacterium than the hit or hits which frees prophage A1c. These findings are in accordance with the conception that the action of u.v. radiation in causing induction is not directly on the prophage, but is on the bacterial host (Jacob, 1954). On the other hand the process is complex, and it is clear that the two prophages, A1b and A1c, despite their close relationship, have each their own specific triggering mechanism, and are activated by different stimuli, the nature of which has still to be discovered.

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The Gram-Staining Reaction of Disintegrated Micro-organisms

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SUMMARY: Disintegration of yeast and *Clostridium welchii* gave cell envelopes which stained Gram-negative by a modified Jensen's Gram-staining procedure (procedure 1), but stained in part Gram-positive by a modification of the Gram stain in which the films were dried after application of the iodine and before application of the decolorizing agent (90% v/v ethanol; procedure 2). Procedure 2 appeared to give a typical Gram reaction in that a series of organisms gave identical results when stained by the two procedures. Disintegrated Gram-negative organisms gave only Gram-negative material when stained by procedure 2. The Gram-positive material could be removed from the yeast envelopes by extraction at pH 8, but attempts to 'replate' the envelopes with the extract were unsuccessful. The results suggest that the Gram-staining reaction of yeast and *C. welchii* is due in part to a specific staining component and in part to a factor dependent upon the intact nature of the cells. The latter may be associated with the stability or orientation of the staining component or with some membrane effect.

A number of workers have noted that when Gram-positive organisms are broken they become Gram-negative (Benians, 1912, 1919; Burke & Barnes, 1929). These results have been taken as support for the theory that the Gram-staining reaction is due to the presence of a membrane in Gram-positive organisms which is impermeable either to the dye+iodine complex or to the decolorizing agent. This effect of breaking cells may be equally well explained as being due to the degradation or disaggregation of a Gram-positive complex when the cell membrane is ruptured. It is not certain that Gram-positive organisms always become Gram-negative when broken. Thus Burke & Barnes (1929) observed certain Gram-positive structures in preparations of broken yeast cells, and Kemp (1931) showed that broken yeast cells showed Gram-positive structures when stained by a particular modification of the Gram stain.

The results reported in the present paper show that although certain Gram-positive organisms which had been disintegrated by shaking with glass balls stained Gram-negative by Jensen's modification of the Gram method, a change in this procedure as simple as just allowing the slide to dry before applying the decolorizing agent resulted in the distintegrated organism staining Gram-positive.

METHODS

Organisms. The following were used: *Saccharomyces cerevisiae* (baker's yeast, Distillers Co., Ltd. DCL); *Clostridium welchii*; *Sarcina lutea*; *Escherichia coli*; *Azotobacter chröococcum* (NCIB 8003); *Caryophanon* sp. (kindly supplied by Dr T. Gibson). *S. lutea*, *C. welchii* and *E. coli* were grown at 37° in a Lemco broth medium, *Azotobacter chroöcoccum* on mannitol agar at 25° and *Caryophanon* sp. on yeast-extract agar at 25°.

Disintegration of the organisms. A suspension of organisms in distilled water (5 ml.) was shaken with ballotini glass balls (no. 5; 3 g.) in a Mickle shaker (Mickle, 1948). Agitation was carried out at full amplitude for periods of 1 min. followed by 1 min. periods at rest, in order to avoid excessive heating. Optimum breakage was found by microscopical examination. The time required varied from 3 min. breaking time for *Caryophanon* sp. to 5–10 min. for baker's yeast.

Techniques used for the Gram-staining method

A small loopful of the fluid containing material to be stained was spread thinly on the slide, allowed to dry in air and then fixed by passing the slide through a Bunsen flame. The reagents used were: aqueous solution of methyl violet 6B (British Drug Houses Ltd.; 0·5 %, w/v); Lugol's iodine solution; 90 % ethanol (ethanol + water, 9 + 1, v/v); dilute carbol-fuchsin (1 in 20 dilution of Ziehl-Neelsen stain).

The film was covered with methyl violet solution which was allowed to act for 2 min. The stain was then poured off and, with the slide held at an angle, the iodine solution was applied so that it washed the slide. The iodine solution was allowed to act for 2 min., then poured off and 90 % ethanol applied for 2 min. The 90 % ethanol was poured off, dilute carbol-fuchsin applied for 15 sec., the slide washed with water and dried. Except for times of application this method approximates to Jensen's modification of the Gram stain and will be referred to as procedure 1.

It was found with disintegrated organisms that different results were obtained when, after treatment with the iodine solution and before decolorization with 90 % ethanol, the slide was dried either by blotting or by allowing to stand in air for a few minutes. This modified method will be known as procedure 2. The best known Gram-staining procedure in which the slide is blotted dry after application of the iodine is that of Hucker (1922). This procedure is recommended by the Society of American Bacteriologists; Burke (1922) recommends blotting but not drying the slide before application of the decolorizing agent. Hucker's procedure was not used in the present work because the short time for the application of the decolorizing agent was not sufficient to remove the dye + iodine complex from the cell debris present in films of disintegrated organisms.

RESULTS

Baker's yeast was disintegrated in the Mickle shaker as previously described and at intervals films prepared and stained by Gram's method. Staining by procedure 1 showed only Gram-negative debris and unstained cell envelopes (Pl. 1, fig. 1), but staining by procedure 2 (Pl. 1, fig. 2) showed, in addition to light purple-staining debris, Gram-positive material, most of which adhered to the cell envelopes. The experiments were also carried out with *Clostridium* *welchii*; preparations stained by procedure 2 (Pl. 1, fig. 3) showed Gram-positive areas. Experiments with *Sarcina lutea* were less conclusive but preparations of disintegrated organisms stained by procedure 2 showed considerably more purple staining than those stained by procedure 1.

In order to eliminate the possibility that the staining was due to artifacts produced by the disintegration of the organisms, the Gram-negative organisms *Escherichia coli*, *Azotobacter chroöcoccum* and *Caryophanon* sp. were disintegrated and stained by procedures 1 and 2. No Gram-positive material was observed.

The possibility that procedure 2 did not give a typical Gram reaction and was capable of giving false positive results was excluded by the fact that procedures 1 and 2 gave identical results when tested on a large number of intact organisms. Thus the following organisms stained Gram-positive by both procedures: *Bacillus megaterium*, *B. subtilis*, *Clostridium welchii*, *Diplococcus crassus*, *Saccharomyces cerevisiae*, *Sarcina lutea*, *Streptococcus faecalis*; and the following organisms stained Gram negative: *Azotobacter chroöcoccum*, *Bacillus brevis*, *Caryophanon* sp. *Chromobacterium prodigiosum* (*Serratia marcescens*), *Cloaca cloacae*, *Escherichia coli*, *Klebsiella aerogenes*, *K. pneumoniae*, *Neisseria catarrhalis*.

The effect of ethanol concentration on the Gram reaction

Since the different results obtained by the two staining procedures must have been due to the presence of water on the slide in procedure 1, the effect of varying the water content of the decolorizing agent on the staining of disintegrated yeast by procedure 2 was investigated. The condition which gave results most similar to those obtained by procedure 1 was with 70 % ethanol (ethanol + water, 7 + 3, v/v), but even in this case there was a slight retention of the purple dye by the cell debris. At lower concentrations of ethanol the debris retained the purple dye and at higher ethanol concentrations the appearance was similar to that obtained with 90 % ethanol. When films of disintegrated yeast were dried after application of the iodine, then re-wetted with water for 2 min. before application of the 90 % ethanol, the films finally obtained were similar to those in which 70 % ethanol was used as the decolorizing agent.

Reversed Gram staining

Attempts were made to stain organisms with an ethanolic solution of the methyl violet + iodine complex. Gram-positive organisms would not be expected to take up the stain if, as has been suggested, they have a cell membrane which is impermeable to the complex. The concentration of methyl violet in a saturated solution of the complex in ethanol was only 0·015 % compared with the 0·5 % for the aqueous solutions used for the normal Gram-staining procedure. Therefore in the former case very poor staining was obtained which could only be detected by macroscopic examination of large films (*c.* 300 cm.^{2}). The technique was to apply the ethanolic solution of the methyl violet + iodine complex to the films for 2 min., remove, dry and wash with absolute ethanol. When the methyl violet + iodine complex was applied in 90 % ethanol, baker's yeast, *Sarcina lutea* and *Escherichia coli* were stained purple, indicating that the dye had penetrated the organisms. It was unlikely that the dye had been only adsorbed on the outer surface of the organisms because the dye could not be removed by washing with absolute ethanol in which the dye + iodine complex is easily soluble, and yeast would not take up dye from a solution of the complex in absolute ethanol. Although the intensity of staining with the 90 % ethanolic solution of the complex was much less than in the direct Gram-staining procedure, this might have been because of the lower concentration of methyl violet which had to be used in the reversed procedure. When yeast was stained with aqueous 0·015 % methyl violet the amount of stain taken up corresponded closely with that taken up from the 90 % ethanolic solution of the methyl violet + iodine complex.

The preparation of Gram-positive cell envelopes from baker's yeast

A suspension of baker's yeast (2 g. fresh pressed DCL yeast in 5 ml. distilled water) was mixed with 3 g. no. 5 ballotini glass balls. The cells were disintegrated as previously described but with cooling in ice water for 2 min. between each 1 min. treatment in the Mickle shaker. When the cells were almost completely broken (5–10 min. breaking time) but the envelopes not destroyed, the suspension (pH 5) was removed from the glass balls, diluted with an equal volume of ice-cold distilled water and centrifuged at 700 *g* in an M.S.E. Minor centrifuge for 5 min. Three layers were observed: an upper layer of liquid, a white middle layer of cell envelopes and a bottom layer consisting mainly of unbroken cells. The middle white layer was separated and washed twice with ice-cold distilled water. This contained cell envelopes which stained Gram positive by procedure 2 (Pl. 1, fig. 4) and was almost free from amorphous debris.

The preparation of Gram-negative yeast cell envelopes

The Gram-positive cell envelopes were suspended in distilled water (*c*. 10 ml.) which had been adjusted to pH 8 with dilute sodium hydroxide. After standing at room temperature for 5 min. the suspension was centrifuged for 3 min. on an M.S.E. Minor centrifuge at 700 *g*. The sediment was re-suspended in distilled water (pH 8, 10 ml.), centrifuged at 700 *g* after 5 min. standing at room temperature and the sediment suspended in distilled water. The cell envelopes in this preparation stained Gram-negative by procedure 2 (Pl. 1, fig. 5).

Extraction of the Gram-positive cell envelopes with 0·1 M-phosphate buffer (pH 8) caused the Gram-positive material to concentrate in the centre of the envelopes (Pl. 1, fig. 6); M-sodium chloride solution had the same effect.

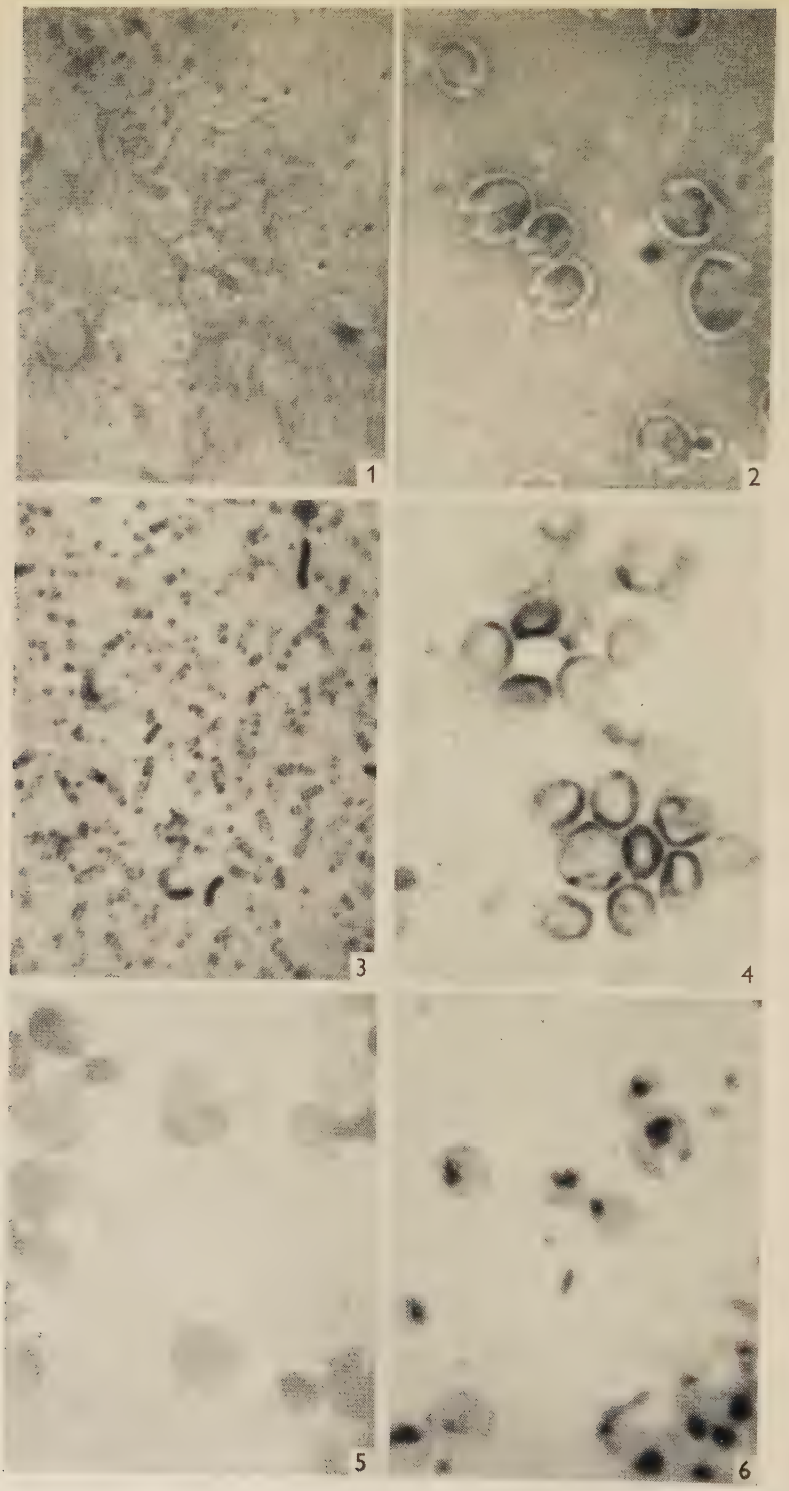
Numerous attempts were made to 'replate' the Gram-negative envelopes with the material extracted at pH 8 and with various other substances such as γ-globulin, ovalbumin, yeast ribonucleic acid, calf thymus deoxyribonucleic acid, and extracts from distintegrated Gram-negative organisms. When the Gram-negative envelopes were suspended in high concentrations of these substances and films made directly, Gram-positive staining material was observed in most cases, but, as the staining property was always lost upon washing the envelopes with distilled water, it was considered that the effect was non-specific and due to the thick films which were obtained by this procedure.

DISCUSSION

The present results render untenable, in the cases of yeast and *Clostridium welchii*, the simple hypothesis that the Gram-positive staining reaction is due to the inability of the dye + iodine complex to diffuse through the cell membranes. If this were the case, it is not apparent why the broken cells should stain Gram-negative by procedure 1 but Gram-positive by procedure 2. It was also shown that methyl violet could penetrate the yeast cell membranes when heat-fixed yeast was treated with a saturated solution of the methyl violet + iodine complex in 90 % ethanol. A direct proof of the permeability of the cell membranes to the methyl violet + iodine complex was not possible by these techniques because of the low solubility of the complex in ethanol. The alternative theory that the decolorizing agent cannot penetrate the cell membranes also does not explain the Gram-positive staining of broken cells by procedure 2, since in this the decolorizing agent could easily penetrate the large gap in the yeast envelopes.

It appears that in yeast and *Clostridium welchii* there is a component which forms a complex with methyl violet and iodine which when dried is not dissociated by 90 % ethanol, but is dissociated by 90 % ethanol when the complex has not dried. It seems that drying the complex forms additional bonds between the dye and the cell component thus rendering the dissociation of the complex more difficult, or that the effect is due to the water on the slide diluting the decolorizing agent. The fact that decolorizing the dried films with 70 % ethanol gave mainly Gram-negative staining supports the latter explanation, although drying the films did fix a little of the methyl violet so that it was not removed even by 70 % ethanol. As films of disintegrated Gram-negative organisms did not stain Gram-positive by procedure 2 it appears that this staining reaction was confined to Gram-positive organisms and indicated the presence of a specific Gram-positive component in yeast and *Clostridium welchii*. The fact that procedure 2 gave a typical staining reaction with a series of intact Gram-positive and Gram-negative organisms is additional evidence in favour of this conclusion. The normal Gram-positive reaction of intact yeast cells, i.e. with a wet slide and 90 % ethanol as the decolorizing agent, may be due either to increased stability of the Gram-positive complex in an intact cell as compared with a broken cell, or to some selection by the cell membranes of the concentration of ethanol which penetrates the cell. That is to say, the Gram reaction in yeast and in *C. welchii* can be attributed in part to a specific cell component and in part to an orientation or membrane effect. The chemical nature of the component in disintegrated yeast which stains Gram-positive by procedure 2 is under investigation.

The authors thank Professor M. Stacey, F.R.S., for his interest in this work.



E. T. J. Chelton & A. S. Jones—Gram staining of disintegrated organisms.
Plate 1

(*Facing p.* 657)

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EXPLANATION OF PLATE

Fig. 1. Film of disintegrated yeast stained by Gram's method, procedure 1 (see text). × 1800.

Fig. 2. Film of disintegrated yeast stained by Gram's method, procedure 2 (see text). × 1200.

Fig. 3. Film of disintegrated *Clostridium welchii* stained by Gram's method, procedure 2. × 1800.

Fig. 4. Gram-positive envelopes obtained by washing disintegrated yeast with distilled water. Stained by procedure 2. × 2200.

Fig. 5. Gram-negative envelopes obtained by washing Gram-positive yeast envelopes with dilute sodium hydroxide (pH 8). Stained by procedure 2. × 2400.

Fig. 6. Gram-positive yeast envelopes treated with 0·1 M-phosphate buffer (pH 8). Stained by procedure 2. × 1800.

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The Death of Micro-Organisms During Drying in Relation to Solute Concentration and Drying Temperature

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SUMMARY: A mixture of *Pseudomonas fluorescens* and coliphage T2 was dried at −20, 0 and +20° in solutions of each of the 16 combinations of mannitol and sucrose at 0, 0·25, 0·50, and 0·75 M. For *P. fluorescens* there was no significant average effect of the drying temperature on the numbers which survived drying, but for coliphage T2 average survival increased as the drying temperature was changed from −20 to 20°. For both organisms, however, there were a number of very highly significant interactions between solutions and drying temperatures. In other words, the effects of the temperature depended on the solutes present and *vice versa*. There were also some substantial differences between the responses of the two organisms. It is concluded that survival during drying is likely to be determined by factors additional to those studied in these experiments.

The most suitable procedures for drying micro-organisms without loss of viability have not yet been defined. There is, however, a widespread opinion that drying from the frozen state gives the best results especially for organisms easily destroyed by drying. For example, Fry (1954) in reviewing results with bacteria stated that 'all the evidence shows that drying from the frozen state is desirable for all organisms and is apparently essential for the more sensitive ones'. Similarly, Harris (1954) suggested that labile viruses should be freeze-dried 'at the lowest convenient temperature'. On the other hand, very many types of micro-organisms show at least a small fraction which survives after a great variety of natural and artificial drying procedures, in many of which drying has undoubtedly occurred at temperatures substantially above 0°. In addition, there have been some comparisons which show that drying above 0° gives greater survival than freeze-drying. Stamp (1947) found that slow drying of *Serratia marcescens* over P_2O_5 at room temperature gave approximately three times as many survivors as either of two methods of freeze-drying. For two types of fungal spores Mazur & Weston (1956) found that spray-drying caused little or no mortality, whereas freeze-drying killed more than 90% of the spores. Greiff, Blumenthal, Chiga & Pinkerton (1954) compared freeze-drying of influenza virus when the ampoules were suspended in baths at 0, −30, −60 and −80°. At −80° and at 0° survival was nearly 100%, but was much less at the two intermediate temperatures. In these experiments of Greiff *et al.* the actual temperatures of the virus suspension were not measured during drying and, as the system was evacuated, the temperature of the product might have been much lower than the temperature of the baths in which the ampoules were immersed. We have found specimen temperatures within evacuated ampoules to be up to 60° below the temperature of the bath surrounding the ampoule. Annear (1956) reported very good survival of various organisms dried rapidly *in vacuo* at temperatures which, although unknown, probably avoided drying from the frozen state. More recently, Annear (1958) extended these observations and found very good survival of *Vibrio cholerae* subjected to fairly rapid vacuum-drying from the liquid state. Annear also found that survival of freeze-dried preparations was generally best when some thawing occurred before drying was complete. As in the experiments of Greiff *et al.* the actual temperatures of the specimens were not measured.

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There is, therefore, conflicting evidence regarding the drying conditions which best conserve viability. While the reason for the disagreement is not obvious, it is of interest to note that different workers have used a variety of experimental conditions, not all of which are independent. As an example, different suspending fluids obviously produce different chemical environments during drying, but they may also give rise to considerable differences both in the rates of drying and in the steady-state temperatures at which drying proceeds. These latter effects result from differences in rates of diffusion of water and water vapour and also, at times, from differences in thermal properties of the specimens. There is, in fact, a rather complicated interdependence of a number of physical and chemical factors which may conceivably affect the biological result.

The experiments now reported were designed to answer two simple questions: (i) is the mortality during drying greatly affected by whether drying is carried out from the frozen or from the liquid state; (ii) are the effects of the drying temperature dependent on the nature of the solutes present? Although there is no particular novelty in this approach the present experiments permitted the actual temperatures obtained in the specimens to be measured and to be partially controlled during drying. The apparatus used was described by Mellor & Ohye (1959).

METHODS

Bacteria were grown in brain + heart infusion broth, aerated by shaking at 30° for 20 hr. Coliphage T2 was propagated on *Escherichia coli* B and a lysate in nutrient broth was filtered and stored at 1°. Suspensions of bacteria were prepared by centrifuging a known volume of broth culture and resuspending the organisms in distilled water at 1/10 the original volume. For the factorial experiment, suspensions of *Pseudomonas fluorescens* prepared in this way were mixed with an equal volume of T2 lysate. One volume of the mixture was then added to 19 vol. of such mixtures of distilled water, mannitol and sucrose solutions as were required to give the desired solute concentrations in the suspensions used for drying. Samples (0·1 ml.) of these suspensions were dispensed in 88 × 9 mm. ampoules and dried in a vacuum drier described by Mellor & Ohye (1959). Drying began within 30–60 min. after the organisms were first transferred to the sucrose + mannitol solutions.

Temperatures of the drying material were measured with 40-gauge thermocouples connected to a recording potentiometer. As the temperatures could be measured concurrently at only 9 points it was not possible to obtain temperature records for each of the 16 solute combinations used in the factorial experiments. Temperatures were, however, measured for the lowest and highest concentrations of each solute, it being assumed that intermediate solute concentrations would probably have temperature histories within the observed range. During drying the temperature of the material was controlled by regulating the temperature of the copper tube-holder, and by adjusting the total pressure inside the vacuum chamber (Mellor & Ohye, 1959). During drying at the nominal temperature of −20° the total pressure in the dryer was not controlled, consequently in the suspension without added solutes most of the sublimation of ice occurred at a much lower temperature than in those suspensions which contained either or both carbohydrates.

On completion of drying all tubes were removed from the dryer and the contents rehydrated immediately with 1·0 ml. nutrient broth. When required further dilutions in nutrient broth were prepared. When mixtures of *Pseudomonas fluorescens* and coliphage T2 were being counted, the former organism was plated on brain + heart infusion agar and incubated at 30° for 48 hr. The phage plaques were developed in a layer of soft nutrient agar seeded with *Escherichia coli* B and incubated at 37° for 24 hr. Under these conditions *P. fluorescens* did not interfere with normal plaque development.

RESULTS

The results of several experiments in which micro-organisms were dried at different temperatures showed that the effect of drying temperature on survival depended on the nature of the solutes present in the suspensions. For several organisms, including *Salmonella newport*, *Pseudomonas fluorescens*, *Vibrio metchnikovi* and the coliphages T2 and T5, survival in 0·5 M-sucrose was very similar whether drying was carried out at *c.* −20° or 0 – 2°, but for suspensions in 0·5 M-mannitol survival was greatly decreased after drying at the lower temperature. There were also suggestions of differential responses between micro-organisms when the drying conditions were changed. Some of these differences between solutes, drying temperatures and organisms have now been confirmed in an experiment which permitted more decisive comparisons.

In this experiment drying was carried out in solutions of each of the possible 16 combinations of mannitol and sucrose at 0, 0·25, 0·50 and 0·75 M. Survival in each of the 16 solutions was measured after drying at each of three nominal temperatures, namely −20, 0 and 20°. The actual temperatures for three of the runs are shown in Fig. 1. In order to provide an estimate of error the experiment was carried out twice at each temperature. As it was possible to dry at only one temperature at one time the whole experiment was divided into 6 blocks, the order in which each block was dried being decided by random selection. In this way there was obtained, for each organism, a total of 96 estimates of the viable counts after drying, the common logarithms of which have been used for the analyses of variance. By drying together a mixture of two organisms which could be enumerated separately, it was possible to obtain evidence about the survival of both organisms and, at the same time, to ensure that any differences in the responses of the two organisms would not be due to environmental differences during drying and rehydration. The two organisms selected were *Pseudomonas fluorescens* and coliphage T2.

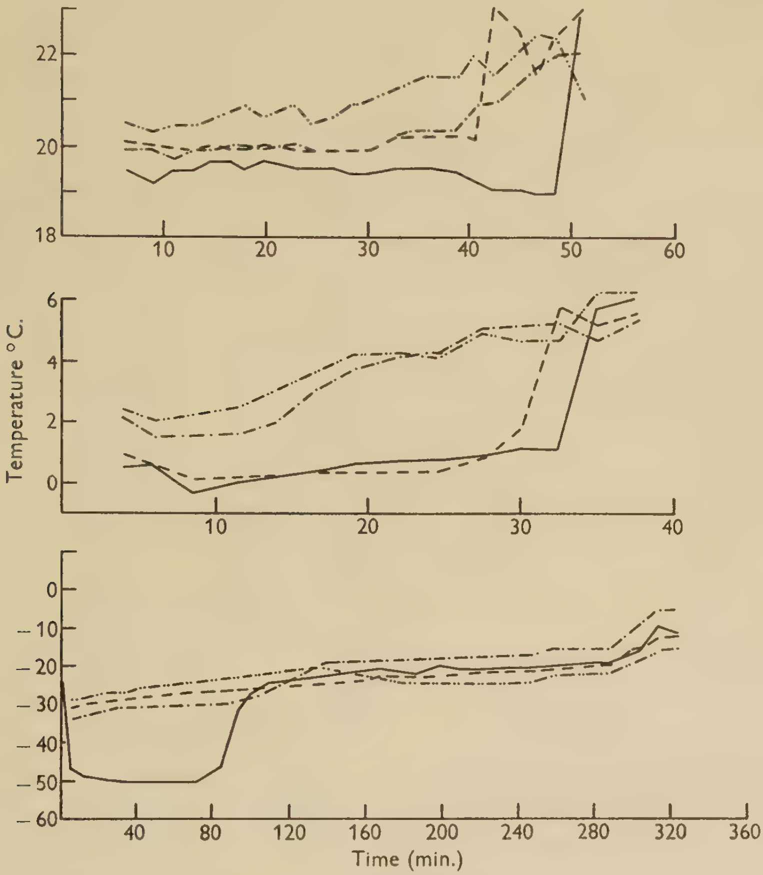

Fig. 1. Temperature changes during drying of coliphage T2 and *Pseudomonas fluorescens* from suspensions containing different proportions of sucrose and mannitol. ———, no sucrose or mannitol; – – – –, mannitol (0·75 M) only; –·–·–·, sucrose (0·75 M) only; –··–··–··, sucrose (0·75 M) and mannitol (0·75 M).

A summary of the results is shown in Fig. 2 and Table 2, together with an abridged analysis of variance in Table 1. (Copies of the complete analyses of variance together with a list of the individual results will be supplied to interested persons on request.) For the purposes of the analysis of variance it was assumed that the three nominal drying temperatures were, in fact, equally spaced; this enabled the linear and quadratic components of temperature to be examined. Similarly, the equal spacing of the sucrose and mannitol concentrations permitted the separation of the linear, quadratic and cubic components for these factors, and of their interactions with temperature. Five of the 96 samples of coliphage T2 yielded no plaques at the highest dilution plated, indicating a viable population of < 100 particles/ml. It was found that when all such counts were assigned the logarithmic value of 2·00, the results would have been almost the same as estimating the missing values. As the principal conclusions appeared to be very clearly established this simpler procedure was adopted.

Table 1. *Drying of coliphage T_2 and* Pseudomonas fluorescens *in sucrose and mannitol solutions at each of three temperatures*

Analyses of variance of log viable counts/ml. after drying.

Source of variation	Degrees of freedom	Coliphage T2 Mean square	Coliphage T2 F	*Pseudomonas fluorescens* Mean square	*Pseudomonas fluorescens* F
Temperature, T	2	15·0998	31·34**	0·9715	1·72
Linear regression on T, Tl	1	29·5392	61·31**	1·9425	3·44
Quadratic regression on T, Tq	1	0·6604	1·37	0·0005	—
Error (whole units)	3	0·4818	—	0·5639	—
Solutions	15	4·5907	8·10***	1·2324	6·41***
Sucrose S	3	0·9388	1·66	5·8362	30·35***
Mannitol M	3	20·4509	36·08***	0·0668	—
Interaction $S \times M$	9	0·5212	—	0·0864	—
Solutions × T	30	4·5727	8·07***	0·7127	3·71***
$Tl \times S$	3	26·2996	46·40***	2·8369	14·75***
$Tl \times M$	3	2·2389	3·95*	0·5076	2·64
$Tq \times S$	3	2·5081	4·43**	0·2378	1·24
$Tq \times M$	3	4·3513	7·68***	0·5328	2·77
$Tl \times S \times M$	9	1·0523	1·86	0·5354	2·78*
$Tq \times S \times M$	9	2·3908	4·22***	0·4686	2·44*
Error (sub units)	45	0·5668	—	0·1923	—

*, **, *** signifies $P < 0{\cdot}05$, $< 0{\cdot}01$, $< 0{\cdot}001$ respectively.

From Table 1 it may be seen that there is a large average effect of temperature for coliphage T2, which is almost completely accounted for by the linear regression. For *Pseudomonas fluorescens*, on the other hand, the average effect of temperature is not significant. Table 2 and Fig. 2 show that the average survival of coliphage T2 was increased as the drying temperature was changed from −20° to 20°. Also from Table 1 it may be seen that there were significant average effects of solutions for each organism, the beneficial effect of mannitol being largely responsible in the case of coliphage T2, and of sucrose in the case of *P. fluorescens*. Consideration of only the average effects, however, is not justified since, for both organisms, the interactions between solutions and temperature are very highly significant.

The large effects of mannitol on the survival of coliphage T2 are mainly linear, being roughly proportional to the concentration (Table 2). Though there is no significant average effect of sucrose for coliphage T2, there is a very large interaction between temperature and sucrose (Tables 1 and 2), drying at 20° giving much better survival at the lowest two concentrations of sucrose (0 and 0·25 M), and −20° giving better results with the highest two concentrations. Sucrose increased the survival after drying at −20° by about 1000-fold, whereas at 0 or 20° its effect was a decrease of 10-fold or 250-fold, respectively. The interpretation of the effects of solutions on coliphage T2 is complicated by the number of significant interactions, especially the highly significant second-order interactions ($Tq \times S \times M$). Table 2 shows that mannitol increased survival of coliphage T2 at all three drying temperatures; drying at −20° gave the smallest survival at all concentrations of mannitol. Figure 2 shows that at 20°, which gave the best average survival of coliphage T2, mannitol had little effect at the lowest two concentrations of sucrose, but was beneficial at the highest two concentrations. Sucrose was deleterious at all concentrations of mannitol for coliphage T2 at 20°, but at 0° it was detrimental only at the higher concentrations and was beneficial in the absence of mannitol. At −20°, sucrose was beneficial at all concentrations of mannitol, while mannitol was beneficial at all concentrations of sucrose.

Table 2. *Solute temperature interactions for coliphage T2 and* Pseudomonas fluorescens *dried at each of three temperatures*

		Coliphage T2				*P. fluorescens*			
Solute	Concn. (M)	−20°	0°	20°	Mean	−20°	0°	20°	Mean
					log viable count/ml.				
Sucrose	0·0	2·69†	6·27	7·48	5·48	7·02	8·13	8·62	7·92
	0·25	4·96	5·56	6·80	5·77	8·91	8·78	9·00	8·89
	0·50	5·93	5·43	5·26	5·54	8·97	8·90	8·93	8·94
	0·75	5·56	5·30	5·02	5·29	9·04	8·84	8·79	8·89
Sucrose and mannitol	Mean	4·78	5·64	6·14	5·52	8·48	8·66	8·83	8·66
Mannitol	0·0	3·33	3·61	5·76	4·23	8·88	8·39	8·80	8·69
	0·25	5·28	5·49	6·01	5·59	8·22	8·65	8·87	8·58
	0·50	5·10	6·36	6·10	5·86	8·55	8·76	8·75	8·68
	0·75	5·42	7·10	6·70	6·40	8·28	8·86	8·91	8·69
Before drying		8·46‡				9·12‡			

† Entries at each temperature and solute concentration are the logarithmic mean of 8 values.

‡ Logarithmic mean of 24 values.

For *Pseudomonas fluorescens*, the effects of sucrose were much smaller than for coliphage T2 even though its average effect was significant for the bacterium (Table 1). The influence of a temperature–sucrose interaction (Table 1) can be seen in the fact that, whereas the absence of sucrose caused the survival of *P. fluorescens* to increase by 40-fold as the drying temperature was increased from −20 to 20°, the presence of sucrose caused the effect of temperature to disappear. Alternatively stated, sucrose solutions increased survival of this organism, dried at −20°, 0° and 20°, by factors of *c.* 100, 6 and 2, respectively (Table 2). Although the temperature–mannitol interactions shown in Table 1 are not significant for *P. fluorescens*, it may be pointed out that the two second-order interactions are significant, as are two of the subdivided interactions (*Tl* × *Mc*; *Tq* × *Mc*) which are not tabulated. The tendency for the effects of mannitol on *P. fluorescens* to be most marked in the absence of sucrose, and to depend also on the temperature (Fig. 2; Table 2) may therefore be real.

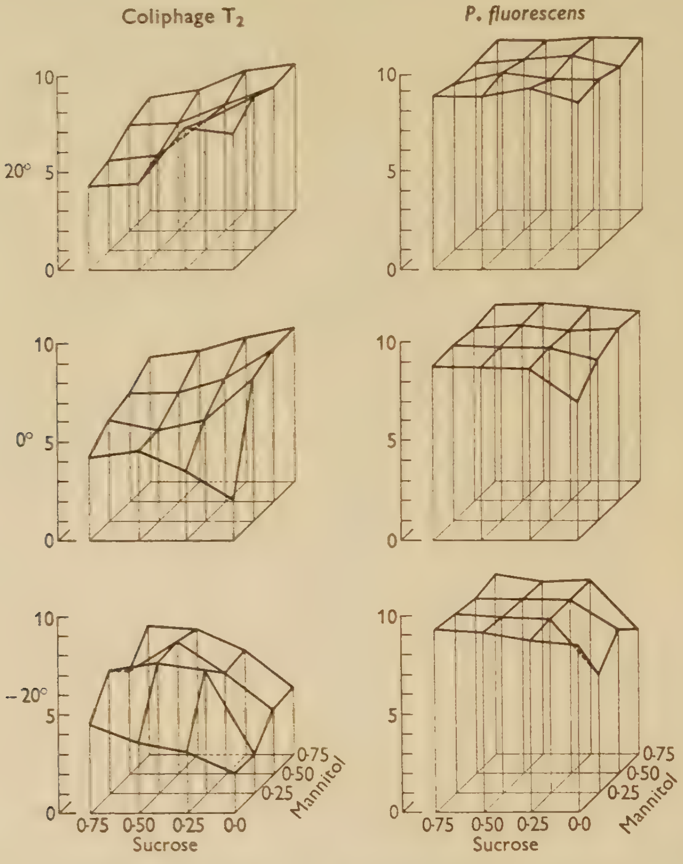

Fig. 2. Solute temperature interactions for coliphage T2 and *Pseudomonas fluorescens* dried in 16 sucrose and/or mannitol solutions at each of three temperatures. Heights of the vertical lines represent the logs of the viable populations/ml. immediately after drying. The scale is shown on the left of each diagram. The concentrations of solutes are given as molarities.

When all drying temperatures are considered (Fig. 2), the best survivals of coliphage T2, which were just over 30 % of the pre-drying counts, were obtained by drying at 0° or 20° in the absence of sucrose and in the presence of mannitol. For *Pseudomonas fluorescens*, several treatments gave survivals which were close to 100 % of the numbers present before drying.

DISCUSSION

In part, the present results support those of some earlier workers who obtained superior survival by drying from the liquid state (Stamp, 1947; Mazur & Weston, 1956; Annear, 1958). In part, they also support the results of those who have reported that drying from the frozen state is either necessary or desirable (Proom & Hemmons, 1949; Hutton, Hilmoe & Roberts, 1951; Fry & Greaves, 1951). The results can also be used to show that sucrose and mannitol do not necessarily produce similar effects, and that either substance may be beneficial or damaging according to the conditions and the test organism. Taken as a whole, however, the results show that the choice of drying temperature depends on the nature of the solutes present. As the results do not support any simple hypothesis about the drying temperature or combinations of sucrose and mannitol which result in the best retention of viability, it is concluded that some additional factors are probably decisive. The nature of the additional factors is, of course, not revealed by the present experiments.

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Specific Exosporium Reaction of *Bacillus megaterium*

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SUMMARY: The sporulation of 51 strains of *Bacillus megaterium* was examined in shaken cultures in potato extract and in Grelet's defined medium. Some abnormal strains are described in which the spores germinate within the sporangia or very soon after their liberation. The spores of *B. megaterium* could be completely freed from vegetative cell remnants by treatment with lysozyme followed by thorough washing. Rabbit anti-sera produced with these treated spores did not cross-react with the vegetative organisms. On the basis of their agglutination reactions the spores of 36 strains of *B. megaterium* were divided into 5 types.

A capsule-like exosporium was detected in wet Indian ink preparations on lysozyme-treated spores of more than half of the 36 strains. The exosporium was rendered visible in the phase-contrast microscope only after the addition of homologous spore antiserum. The exosporium of *Bacillus megaterium* consists of a capsule-like slimy substance which does not derive from the sporangium but which is probably produced during spore formation. The specific exosporium and spore coat reactions are described.

The exosporium was regarded by de Bary (1885) as a pale, weakly refractile, gelatinous structure, either capsule-like or appearing as a polar appendage which forms the outermost layer of the bacterial spore. This opinion was shared by Klein (1889), who observed that even in a dense spore suspension, contiguous spores cannot be seen when the exosporium is well developed. On this basis he rejected the idea that the exosporium is merely a refractile zone. More than half a century later, in his comprehensive review on the cytology of the bacterial endospore, Knaysi (1948) stated that the outer spore coat may be a slime layer. He sometimes designates this as exosporium but considers that its presence is debatable. In the remarkable electron microscopic study made by Knaysi & Hillier (1949), in which parallel beaded fibres were discovered in the spore coat of *Bacillus megaterium*, no trace of exosporium was found. A voluminous outer coat or giant capsule surrounding the main spore wall and corresponding to the exosporium of de Bary was first observed in the sections of spores of *B. cereus* in the electron microscope by Robinow (1951, 1953) and by Chapman (1956). No such structure was observed, however, in sections of spores of *B. megaterium*, although in this species the spore coat proper consists of two membranes lying close to one another, in contrast to the single layered wall of *B. cereus* (Robinow, 1951, 1953; Chapman, 1956; Mayall & Robinow, 1957). An exosporium was also observed by Dondero & Holbert (1957) in ultra-thin sections of *B. polymixa*.

This brief review of the literature on the exosporium is necessarily incomplete, but it shows that the observations have been contradictory even in a relatively small number of species. In recent years the examination of ultra-thin sections in the electron microscope has given considerable information on the internal structure of the spore coat, but with this method it has not been possible to detect whether the spores of *Bacillus megaterium* are surrounded by a slime layer or whether the equivalent of an exosporium is completely lacking in this species (Chapman, 1956). No progress has been made in the last 70 years about the derivation of the exosporium which, as pointed out by Robinow (1953), has been considered by some workers as a condensation of the bacterial cytoplasm. Chapman (1956) regarded even the outer spore coat as a condensation of sporangial cytoplasm. Since Defalle (1902), Mellon & Anderson (1919), Howie & Cruickshank (1940) and Lamanna (1940*a*, *b*, 1942) clearly showed that the vegetative antigens are serologically distinct, we felt that it might be possible to solve the problem of the derivation of the exosporium by the use of immunocytological methods (Tomcsik, 1956*a*, *b*). *B. megaterium* was selected for systematic study of the 'specific exosporium reaction' for two reasons: (i) because the exosporium of this species cannot be detected in the electron microscope; (ii) because the vegetative organisms can be easily digested with lysozyme (Baumann-Grace & Tomcsik, 1957*a*) in contrast to most other species of *Bacillus*, thus providing an easy and reliable method for obtaining spores free from vegetative remnants.

METHODS

Organisms. Fifty-one *Bacillus megaterium* strains previously described (Baumann-Grace & Tomcsik, 1957*b*) were used.

Media. The potato media used by Behring (1889) for sporulation, and recommended on the basis of careful studies for the same purpose by Schreiber (1896), was more recently described by Robinow (1951). Following the recommendation of Powell & Strange (1956) 1/10 volume of Gladstone & Fildes broth (1940) was sometimes added.

A defined medium used by Grelet (1950, 1951) for studies on the sporulation of *Bacillus megaterium*, in which the sole sources of carbon and nitrogen are glucose and nitrate, respectively, was also used. The medium was prepared according to Grelet except that the glucose was decreased from 4·5 to 0·5% (w/v). The final pH value of the medium was 5·8. Some of our strains did not grow at this pH value and required adjustment to pH 6·8. Other strains required the addition of 0·5% (w/v) asparagine. Liquid cultures in potato and Grelet media were obtained by rocking in ⊥ tubes (Kay & Fildes, 1950).

Lysozyme treatment. In order to free the spores from remnants of vegetative organism, the sporulating cultures were centrifuged and the deposit resuspended in the same volume of M/30 phosphate buffer (pH 7·0). An equal volume of buffer containing 1/10,000 dilution of crystalline lysozyme (Armour) was then added and the suspension incubated at 37° for 30 min. or until no vegetative remains were detectable microscopically. The spore suspensions were then chilled immediately and washed 5 times with distilled water in the cold. Previous observations had shown that, on the basis of the lysozyme sensitivity of young vegetative organisms, strains of *Bacillus megaterium* can be divided into two groups (Baumann-Grace & Tomcsik, 1957*a*). In the present work lysozyme at 1/20,000 (w/v) was usually sufficient to remove the vegetative remnants from the sporulating organisms of both groups within 30 min.

'*Vegetative*' *anti-sera* were mostly those used previously (Baumann-Grace & Tomcsik, 1957*b*). They were prepared from 16 hr. cultures on Gladstone & Fildes agar, which is unfavourable for sporulation.

'*Spore*' *anti-sera.* Moderately thick suspensions of lysozyme-treated spores were washed 5 times with distilled water and freeze-dried in 2 ml. volumes. The spores were resuspended in 2 ml. saline for each injection. Rabbits were inoculated intravenously with 6–8 doses at 3- to 4-day intervals between each dose and were bled 7–8 days after the last injection. The resulting antisera agglutinated at a dilution of 1/640 or higher.

Spore agglutination. For this purpose the centrifugation method was used, the advantage being that the results are easier to read and incubation at 37°, which might lead to germination of the spores, is avoided. The tests were set up in the usual way with 0·2 ml. serum dilution and 0·2 ml. spore suspension and incubated at 4° for 30–60 min. The tubes were then centrifuged immediately for 4 min. and the results read by gently tapping the tube to shake up the deposit.

Specific sporangium reaction. This was carried out with 'vegetative' antisera as described previously (Tomcsik & Baumann-Grace, 1958).

Specific exosporium reaction. (*a*) One loopful of undiluted serum was applied to the edge of the coverslip of a wet mount of unstained spores and allowed to flow under the coverslip by capillary action. The preparations were examined by phase contrast. (*b*) Preparations obtained by mixing one loopful of spore suspension with antiserum on a slide and covering with a coverglass. This second method was used for the systematic examinations when each suspension was examined with 8 or more spore antisera. Readings taken immediately or after storing the preparations for several hours in a wet chamber were essentially the same, and were only complicated in a few instances by germination of the spores in serum mixture after a few hours at room temperature. Too high a concentration of spores in the suspension occasionally led to negative results; for instance strain Mg 29 gave a negative exosporium reaction when the optical density of the suspension was greater than $BaSO_4$ standard (McFarland) no. 4.

RESULTS

Production of spores and their purification from vegetative cell remnants

Since it was found that the growth and sporulation of several strains of *Bacillus megaterium* were as good in potato extract in rocked ⊥ tubes at 37° or 28° whether or not 1/10 vol. Gladstone & Fildes (1940) broth was added, spore production was usually carried out in simple potato extract. Twelve out of 51 strains were asporogenous. This high proportion may be due to the fact that the strains have been subcultured monthly on Gladstone & Fildes agar since we isolated or received them about three years ago. This medium was unfavourable for sporulation (Tomcsik & Baumann-Grace, 1958) and may therefore have favoured the production of asporogenous varieties. Of the remaining 39 strains, 29 sporulated rapidly in potato medium and showed 80–100 % spores which began to be liberated in 16–17 hr. at 28°. Compared with potato extract, maximum growth, spore production and liberation of the spores were obtained about 24 hr. later on Grelet's defined medium, even after re-incubation and addition of 0·5 asparagine. The most favourable time for harvesting the spores was at 23–24 hr. in Grelet's medium.

A few strains behaved unexpectedly in that abundant sporulation occurred only on potato agar and not in the shaken liquid medium. The 'M' strain (Tomcsik & Guex-Holzer, 1951) was most noticeable in this respect; only 1–2 % spores were formed after 3–4 days in shaken potato or Grelet's medium, but on potato agar almost 100 % sporulation was obtained in 2 days. The only further abnormality found in the sporulation of this strain was that no liberation of the spores took place. Even after incubation for 10 days there was no sign of disintegration of the long chains. Addition of glutamyl-polypeptide antibody or specific cell-wall antibody showed that the surface structures of the sporangia were still intact. The spores were completely liberated by lysozyme and remained refractile when stored at 4°.

Another phenomenon unknown to us was displayed by 8 of our strains (Mg 2, 11, 16, 21, 22, 27, 29, 40). Although typical refractile spores were found in the shaken potato cultures after 15–16 hr., they did not remain so but were rapidly converted, often within an hour or less, into large black ovoid bodies. Depending on the strain, 10–100 % of the organisms in the culture consisted of these elements. Seen in the phase-contrast microscope they resembled spores in the first stage of germination as described by Pulvertaft & Haynes (1951). Since these bodies were resistant to lysozyme they may repersent incompletely germinated spores. Their production may be a hereditary property; for example, strain Mg 11 formed these black bodies regularly whether cultured in potato or in Grelet medium. An incomplete stage in spore production is shown in Pl. 2, fig. 5, in which most of the cellular elements consist of these ovoid structures. Contrary to their usual appearance, however, these structures are pale, suggesting some form of lysis. As can be seen from Pl. 2, fig. 7, these structures were resistant to lysozyme, whereas the vegetative organisms were completely dissolved. Addition of homologous vegetative antiserum to a lysozyme-treated washed suspension revealed no vegetative remnants.

Immunologically specific reactions on the surface of Bacillus megaterium *spores*

On examination of lysozyme-treated washed spores in wet indian ink preparations, no trace of an exosporium was seen in more than half the strains; in the remainder the spores were surrounded by a distinct capsule-like zone into which the indian ink particles did not penetrate. With 9 of the strains the visibility of this structure was considered to be very good (illustrated in Pl. 1, fig. 1). The preparations were made by inverting the cover-slip on to the mixture of indian ink and organisms and gently pressing it down with filter-paper. By increasing the pressure, the spores lost their refractility and increased in size, appearing as large black elements. These structures still retained their exosporium (see Pl. 1, fig. 2), although it was somewhat decreased in size. Despite the similarity in morphology between the black elements formed by crushing the spores and those produced by cultures of strain Mg 11 (Pl. 2, fig. 5), there is no relationship between the two.

An attempt was made to demonstrate the capsule-like exosporium in the phase-contrast microscope by using specific spore antiserum. Spore antisera against eight different strains of *Bacillus megaterium* were made; these agglutinated the homologous spores at a dilution of 1/64 and usually in 1/2560. The vegetative organisms were, however, not agglutinated in 1/20 dilution of the spore antisera and no specific cell-wall reaction could be elicited. After mixing one loopful of spore suspension, of a strain which showed a well-defined exosporium in indian ink, with one loopful of homologous spore antiserum, the exosporium became distinctly visible (Pl. 1, fig. 4). This reaction was specific; no trace of exosporium was seen without addition of antiserum or when homologous 'vegetative' or heterologous spore antiserum was substituted for homologous spore antiserum. For this reason the reaction was designated as the 'specific exosporium reaction', since it appeared to be similar in nature to the specific capsular reaction (Tomcsik, 1956*b*). This reaction was also carried out with a sporulating suspension of strain Mg11 before (Pl. 2, fig. 6) and after (Pl. 2, fig. 8) lysozyme treatment. Although addition of homologous 'vegetative' antiserum gave a strong vegetative cell-wall reaction with isolated cell walls of strain Mg11, its interaction with the intact bacilli was hardly visible (Pl. 2, fig. 5). No reaction was detected on the intact spores or on incompletely germinated spores, whether intact or partially empty (Pl. 2, figs. 5, 7). The last-mentioned elements, however, gave a specific spore-coat reaction on adding specific spore antiserum (Pl. 2, figs. 6, 8) and a weak exosporium reaction was observed on the refractile spores, although the latter can only be seen clearly with a magnifying glass (Pl. 2, fig. 8). Spore walls of other strains obtained by shaking intact spores in the Mickle disintegrator also showed a specific spore-coat reaction on adding homologous spore antiserum. This reaction is considered to be analogous to the specific cell-wall reaction (Tomcsik & Guex-Holzer, 1954).

Serological types of Bacillus megaterium *spores*

Spores of 4 strains selected at random (Mg 19, 45, 11, 17) were used to obtain our first spore antisera in rabbits. By cross-agglutination tests with the resulting antisera it was found that they represented 4 distinct spore types which showed no cross-reaction with one another. By agglutination tests between these antisera and spores of the remaining 35 strains, 24 were found to be agglutinated by one or other of the antisera. Of the remaining 11 strains which could not be typed, 4 (Mg 1, 6, 35 and 39) were again selected at random for rabbit immunization. However, contrary to the results with the first

4 antisera produced, the later antisera gave cross-reactions to some extent, but only 3 strains could not be agglutinated with one or more of the 8 spore antisera obtained. The specific exosporium reaction was always carried out before the agglutination test in order not to be influenced subjectively by knowing the results of the latter. Since it is difficult to evaluate this morphological reaction in strains which have a poorly developed exosporium, the initial readings were always carried out by one of us (J.T.).

Table 1. *Serological types of* Bacillus megaterium *spores established with agglutination and with specific exosporium reactions*

Spore type	Spores used for immunization	Agglutination*	Specific exosporium reaction	Exosporium visibility in indian ink		
				Negative or indistinct	Distinct	Very good
A	Mg 19	+ +	Distinct	.	2†	19, 25, 27, 30a†
A	Mg 19	+ +	Weak	9, 12, 13, 29	M	.
A	Mg 19	+ +	Negative	20, 41, 46, 51	.	.
A	Mg 19	+	Negative	48	.	.
B	Mg 45	+ +	Distinct	.	40, 45, 47	16, 21, 24, 31
B	Mg 45	+ +	Weak	37, 48	36	.
C	Mg 11	+ +	Distinct	.	11	—
D	Mg 17	+ +	Weak	17	.	—
D	Mg 17	+ +	Negative	53	.	.
E	Mg 1, 6, 35, 39	+ +	Distinct	.	6, 35	42
E	Mg 1, 6, 35, 39	+ +	Weak	39	22	.
E	Mg 1, 6, 35, 39	+ +	Negative	1, 3, 8	.	.

* Agglutination: + positive in dilution 1/100; + +, positive in dilution 1/160 or more.
† No. of *B. megaterium* strain in our collection.

The results of the spore typing on the basis of the specific exosporium and agglutination reactions are summarized in Table 1. To facilitate the discussion, the first 4 spore types have been designated A, B, C, and D. Spores of 15 strains were grouped as A, 10 as B, 1 as C and 2 as D. The last spore type, E, contains several subtypes, some being related to group D; their complicated cross-relationship is summarized in Table 2. Table 1 also shows that the specific exosporium reaction can be used for serological typing when the exosporium is sufficiently well developed as judged by indian ink preparations. On the whole, it was found that the visibility of the exosporium in indian ink preparations was best with type B spores; the exosporium was correspondingly good with these strains.

The majority of the strains of the small-cell variety of *Bacillus megaterium* (Baumann-Grace & Tomcsik, 1957*b*) were asporogenous, but those which formed spores belonged entirely to type A. Some of the large-celled varieties, however, also belong to this group.

Table 2. *Selected tests comparing the strict specificity of* Bacillus megaterium *spore types A, B, C, with the irregular cross reactions of types D and E*

Strain no.	Exosporium visibility in indian ink	Spore serum types							
		A	B	C	D	E			
		19	45	11	17	1	6	35	39
Mg 19	Very good	3, + + +	–	–	–	–	–	–	–
Mg 45	Distinct	–	2, + + +	–	–	–	–	–	–
Mg 11	Distinct	–	–	2, + + +	–	–	–	–	–
Mg 17	Indistinct	–	–	–	1, + + +	–	–	–	–
Mg 3	Negative	–	–	–	–	–, + + +	–	–	–
Mg 13	Negative	–	–	–	–	–, + +	–, + + +	–, + + +	–, 0
Mg 1	Indistinct	–	–	–	–	–, + +	–	–	–
Mg 39	Indistinct	–	–	–	–	–	–	–	1, + +
Mg 6	Distinct	–	–	–	1, +	–	2, + +	2, + +	2, + +
Mg 22	Distinct	–	–	–	2, + +	–	1, + +	1, + +	1, + +
Mg 35	Very good	–	–	–	–	–	–	2, + + +	–
Mg 42	Very good	–	–	–	2, + +	–	2, + + +	1, + + +	2, + +

–, specific exosporium reaction, or agglutination negative; 1, specific exosporium reaction weak; 2, specific exosporium reaction distinct; 3, specific exosporium reaction strong; +, agglutination in 1/100 antiserum dilution; + +, agglutination in 1/160–1/320 antiserum dilution; + + +, agglutination in 1/640 antiserum dilution or more; 0, not examined.

DISCUSSION

In the present work the term exosporium has been given to the slimy, capsule-like outer layer which was observed on lysozyme-treated washed spores of more than half of our strains of *Bacillus megaterium* in wet indian ink preparations. A description of a similar structure is to be found in de Bary's book (1885), but he gave no name to it. The term exosporium was first used by Flügge (1886) and since then by many authors to designate widely different substances or membranes on the surface of the spores. We have, however, kept the term exosporium for *B. megaterium* spores, instead of inventing a new expression, in order to avoid increasing the confusion in the nomenclature of the various spore layers.

The capsule-like structure described here does not appear to correspond to either of the spore membranes found in ultra-thin sections of *Bacillus megaterium* spores. Robinow (1951, 1953) did not find an exosporium on spores of this species and Chapman (1956) stated even more categorically that *B. megaterium* lacks an exosporium. Our observations, however, suggest that although the exosporium of *B. megaterium* can be demonstrated by the very simple indian ink technique, it is most probably of a slimy nature and therefore not suitable for detection by electron microscopy.

According to Robinow (1953) most investigators believe that the exosporium is derived from the vegetative cell; Chapman (1956) considered even the outer spore coat to be a condensation of bacterial cytoplasm. We have been able to show with certainty that the capsule-like exosporium in *Bacillus megaterium* is a specific spore substance and is not derived from the vegetative cell.

Mellon & Anderson (1919) demonstrated that the spore and vegetative stages in *Bacillus subtilis* are serologically distinct, but also pointed out the difficulties of producing exclusively spore antisera unless the disorganized remnants of the vegetative cells are removed from the spore antigen used. Howie & Cruickshank (1940) and Lamanna (1940*a*, *b*) were apparently able to overcome these difficulties. With *B. megaterium* we found that this problem could be easily solved since the vegetative cells of this species are susceptible to lysozyme (Baumann-Grace & Tomcsik, 1957*a*, *b*), whereas the spores are completely resistant. Treatment of sporulating cultures with lysozyme and subsequent washing appears to remove all traces of 'vegetative' antigens since no 'vegetative' antibodies could be detected in the resulting spore antisera and the treated spores did not react with the specific 'vegetative' antisera. We found with the 'specific exosporium reaction' that the slimy, capsule-like exosporium of *B. megaterium* can be regarded as corresponding to the outermost layer of the spore and not to vegetative cell material. A similar reaction, which was designated the 'specific spore coat reaction' was observed on the empty spores. Like the specific cell-wall reaction, the specific spore coat reaction cannot be demonstrated on intact organisms. For this purpose the spore coats must be separated from the spore contents either by shaking in the Mickle disintegrator or by making use of empty, abnormally germinated spores, before the reaction can be observed. A similar reaction which has been found on the spores of *B. cereus* will be described fully elsewhere.

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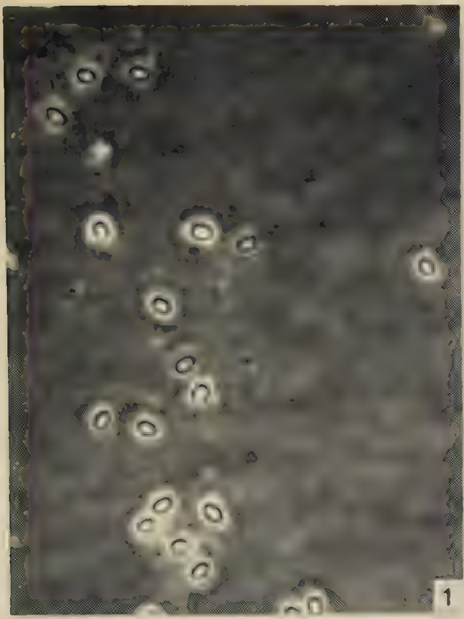
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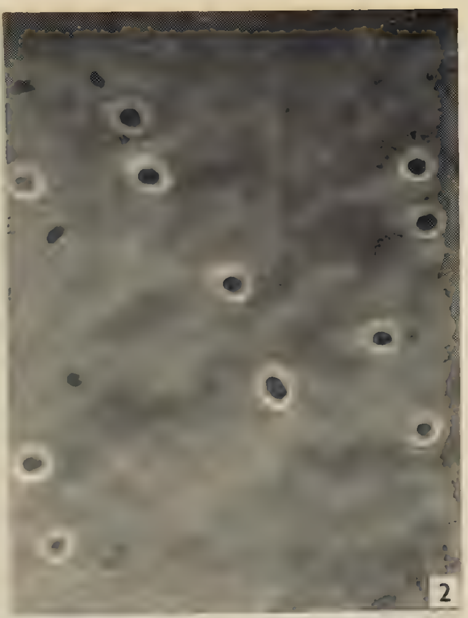
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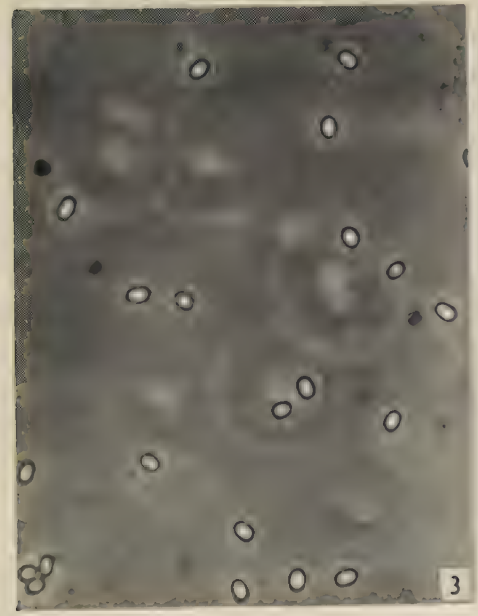
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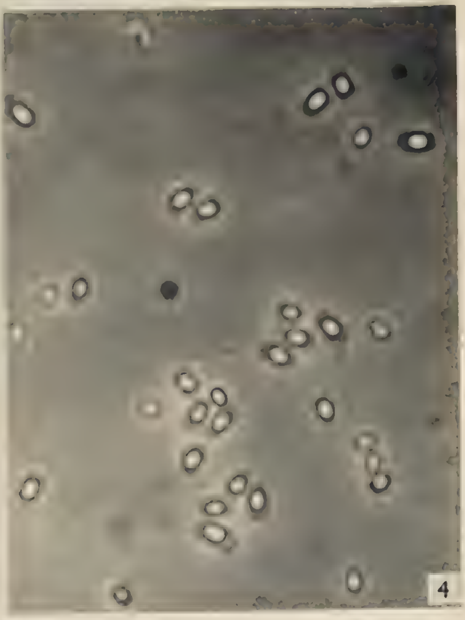
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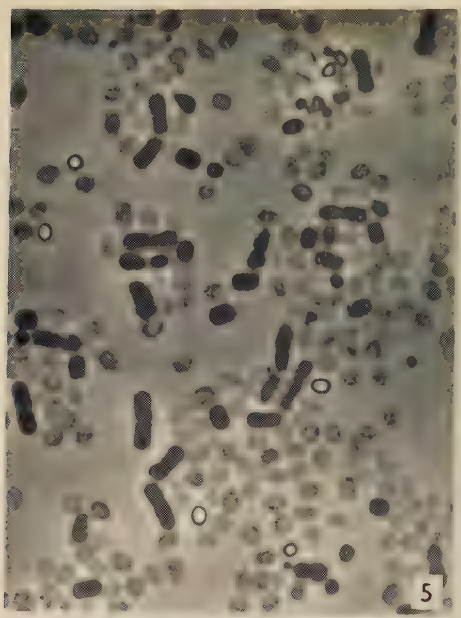
J. TOMCSIK & J. B. BAUMANN-GRACE SPECIFIC EXOSPORIUM REACTION. PLATE 1

(*Facing p.* 674)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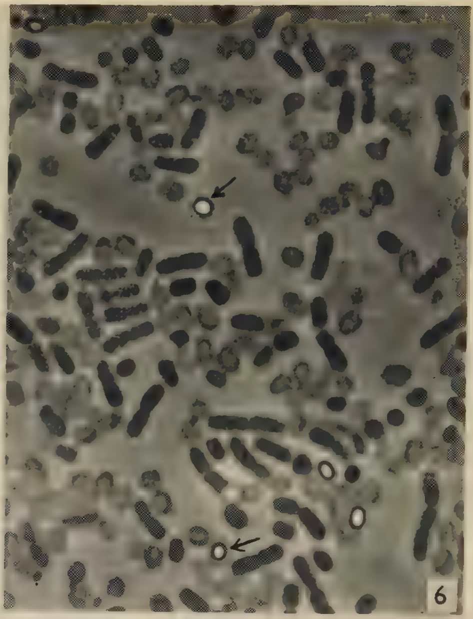
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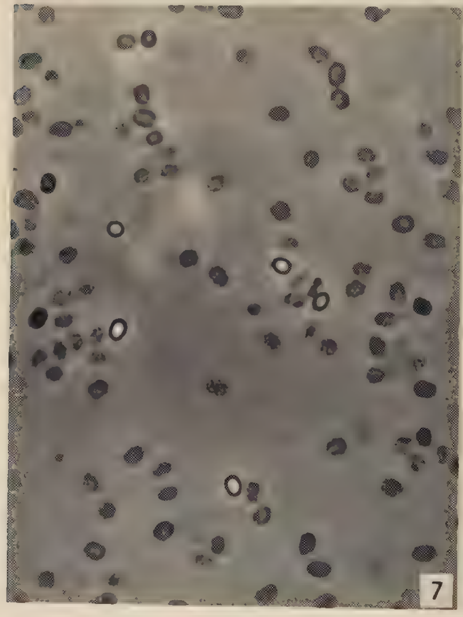
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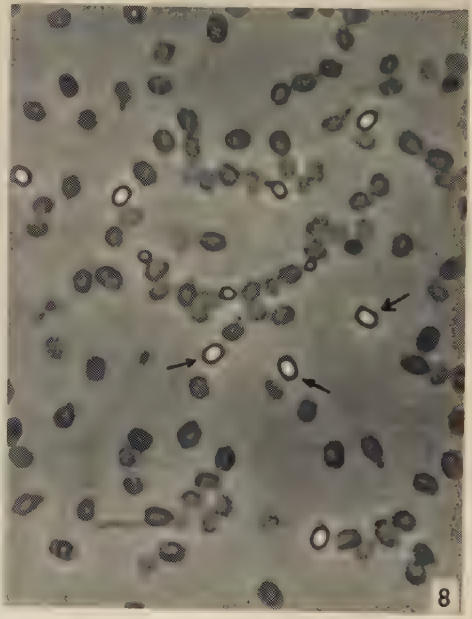
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J. TOMCSIK & J. B. BAUMANN-GRACE—SPECIFIC EXOSPORIUM REACTION. PLATE 2

Robinow, C. F. (1953). Spore structure as revealed by thin sections. *J. Bact.* **66**, 300.

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EXPLANATION OF PLATES

Phase-contrast photomicrographs of *Bacillus megaterium* spores. Magnification *c.* ×2500.

Plate 1

Fig. 1. Strain Mg 42; 24 hr. potato extract culture after dissolution of vegetative cell remnants with lysozyme. Wet indian ink preparation showing large, capsule-like exosporium on typical refractile spores.

Fig. 2. Strain Mg 45; preparation as fig. 1. The coverslip has been firmly pressed down, crushing the spores which appear as black ovoid bodies surrounded by an exosporium.

Fig. 3. Strain Mg 24; 38 hr. shaken culture from neutralized Grelet medium + 0·5 % asparagine after treatment with lysozyme. Wet mount in physiological saline showing refractile spores surrounded by a diffraction zone. No exosporium visible.

Fig. 4. Same spore suspension as fig. 3 after addition of type B spore antiserum, showing a typical specific exosporium reaction.

Plate 2

Fig. 5. Strain Mg 11; 40 hr. shaken culture from Grelet medium (pH 5·8) + 0·5 % asparagine after the addition of specific 'vegetative' antiserum Mg 11, showing many intact vegetative organisms, 5 refractile spores and numerous empty black ovoid bodies which do not react with the antibody. Wet mount in saline.

Fig. 6. A similar preparation as in fig. 5 except that specific spore antiserum has been added instead of 'vegetative' antiserum. A specific spore coat reaction can be seen on the black ovoid bodies.

Fig. 7. The same suspension as in fig. 5 after total elimination of the vegetative organisms with lysozyme, and addition of 'vegetative' cell antiserum Mg 11.

Fig. 8. The same suspension as in fig. 7 to which strain Mg 11 spore antiserum has been added in place of 'vegetative' antiserum. A specific spore coat reaction can be seen on the black ovoid bodies and a weak exosporium reaction on the refractile spores.

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Conditions for the Infection of *Escherichia coli* with Lambda Phage and for the Establishment of Lysogeny

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SUMMARY: Optimal conditions for the adsorption of bacteriophage λ_{22} by *Escherichia coli* strain K112 require 0·02 M-$MgSO_4$ and a pH value of the order 6·5. With a multiplicity of input of 20 phage particles per bacterium, 99 % of the bacteria are infected and at least 90 % are lysogenized. Irradiation of the cells with ultraviolet light immediately prior to infection causes them to give the lytic rather than the lysogenic response.

When a bacterial cell is infected with a temperate phage, there are two major characteristic types of response, the lytic and the lysogenic. In the lytic response, the phage passes into the vegetative state, and a large number of infective particles are eventually synthesized and the cell lyses. In the lysogenic response, the genome of the virus becomes attached to the bacterial chromosome and thereafter this bacterium and its descendants are endowed with the ability to produce, in certain circumstances, infective phage particles similar to those involved in the original infection. At the present time, little is known about the processes which decide whether a cell will give a lytic or lysogenic response, and there is no detailed information about the sequence of events between infection and the establishment of lysogeny. Lieb (1953) was the first to make a comprehensive study of the establishment of lysogeny by λ phage in *Escherichia coli*. In her experiments, the phage and cells were mixed in a broth medium, consequently several processes such as the adsorption of the phage, infection, development of vegetative phage and the establishment of the prophage were all going on at the same time in different cells. With biochemical studies as the ultimate aim, it was desirable to achieve infection of *E. coli* with λ phage in a simple mineral medium and then transfer the infected bacteria to a complete growth medium: in these conditions all the infected cells should begin to respond at about the same time. Kaiser (1957) has reported good adsorption of λ phages to *E. coli* in the presence of 0·01 M-$MgSO_4$. With *E. coli* K112 and λ_{22}, adsorption in 0·01 M-$MgSO_4$ was relatively poor, and often less than 50 % of the cells were infected. However, the concentration of $MgSO_4$ has a profound effect on the total number of bacteria which can be infected and conditions were eventually found in which about 99 % of the bacteria were infected, about 90 % giving the lysogenic response. A preliminary report of these experiments and the effect of the establishment of lysogeny on the metabolic activities of the cells has been made (Fry & Gros, 1957).

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METHODS

Organisms. Escherichia coli strains K112, HfrC, (λ_{22}) (lysogenic for λ_{22} and source of phage stock) and C600 Sr^r (streptomycin resistant and indicator bacteria for λ) were from the culture collection of Drs F. Jacob and E. Wollman of the Pasteur Institute, Paris.

Growth media and assay methods. The media and plating procedures were based on those of Adams (1950). Nutrient broth (medium A) contained 1% (w/v) peptone, 1% (w/v) Liebig's meat extract and 0·5% (w/v) NaCl. Medium B for plating procedures was similar to medium A, but with 1% (w/v) NaCl and agar as required (2% (w/v) for colony counts; 1% (w/v) for assay of phage and detection of cells giving a lytic or lysogenic response; 0·7% (w/v) for small tubes in which samples were dispersed prior to plating). For the detection of lysogeny, medium B was supplemented with lactose (2%, w/v) and Prolabo Universal Indicator (5 ml./100 ml.), thus giving a green coloured medium (medium C, cf. Kaiser, 1957). The strains of *Escherichia coli* K112 and HfrC used in these experiments can utilize lactose and when plated on this green medium the resultant colonies are stained yellow and are readily visible. Consequently, when λ-infected cells of strains K112 and HfrC are plated with the indicator bacteria (free phage having been removed by anti-λ serum), it is easy to distinguish those cells giving a lytic response (large turbid plaques), cells not affected by the experimental procedures—refractory or resistant cells (yellow colonies) and cells giving a lysogenic response (formation of yellow colony surrounded by halo of lysis due to release of λ by spontaneous induction in a few cells during growth of the colony). After overnight incubation of the plates at 37°, the plaques from the cells giving the lytic response are fully formed, but a further 24 hr. at 37° is required for all the lysogenic cells to give well-defined haloes round the colonies. In trial experiments with *E. coli* strain K112 (λ_{22}) at least 95% of the cells gave haloed colonies in these conditions. In general, conditions were arranged such that the responses of about 100 cells were tested on each plate. All platings were done in triplicate and results expressed as the average of the three plates.

Preparation of phage stocks. Escherichia coli strain K112 (λ_{22}) was grown with gentle shaking in medium A at 37° for 3 hr. to $1{\cdot}5$–2×10^8 cells/ml. The cells were collected by centrifugation, suspended in sterile distilled water (2–4×10^8 cells/ml.) and then irradiated with ultraviolet (u.v.) light (from a G.E.C. germicidal lamp giving an intensity of illumination at 2570Å. of approx. 500 ergs/sq.mm. at a surface 1 m. below the lamp): the amount of irradiation was sufficient to bring about at least 95% induction. The suspension of irradiated cells was mixed with one-fifth of its volume of medium A and then shaken at 37° until optical density measurements showed that lysis was nearly complete. Bacteria were removed by centrifuging at 3000 *g*. The supernatant was centrifuged at 20,000 *g* for 1 hr. in a Spinco centrifuge and the pellet resuspended in 20% medium A in distilled water (debris was removed by centrifugation at 3000 *g*). These phage stocks (about 3×10^{11} particles/ml.)

were stable for several months at 4°: $CHCl_3$ was added to prevent bacterial contamination.

Preparation of cell suspensions. Cells of *Escherichia coli* in the late log phase of growth adsorb λ phage well (Lieb, 1953) and such cells were used in the experiments described below. Cultures in medium A were incubated in Ehrlenmeyer flasks (50 ml./500 ml. flask) shaken in a water bath at 37°. After about 4 hr., when the cell density was 3–4 $\times 10^9$ cells/ml., the bacteria were collected by centrifugation, washed twice in water and suspended in distilled water at a concentration of 2×10^8 cells/ml.

Measurement of phage adsorption. Cells of strains K112 or HfrC (both strains sensitive to streptomycin) were mixed with phage in the required adsorption medium; samples were taken at intervals, and after appropriate dilution in broth, 0·1 ml. was plated with streptomycin (0·05 ml. of a solution containing 100 mg./ml.) and streptomycin-resistant indicator bacteria (Bertani, 1951). This method enables the unadsorbed phage at the time of sampling to be readily determined.

Detection of type of response of cells after treatment with phage. At the end of the time allowed for adsorption, free phage was removed by diluting into anti-λ serum in broth (1/200, v/v). After 10 min. at 37°, a sample was diluted in broth and incubated at 37° for a further 10 min. Appropriate amounts were then taken for plating with the indicator bacteria, *Escherichia coli* C600 on medium C.

Terminology. 'Multiplicity of exposure' refers to the average number of phage per bacterial cell in the adsorption tube at the beginning of infection. In order to calculate the 'multiplicity of infection', the total number of phage adsorbed by a given number of cells was determined by experiment and it was assumed that cells giving the refractory response (i.e. neither lytic nor lysogenic responses) did not adsorb phage. The concentrations of cells refer to viable counts determined after dilution in medium A and plating on medium A plus 2% (w/v) agar.

RESULTS

Effect of concentration of $MgSO_4$ *on type of response to infection with* λ_{22} *phage*

Samples of the same washed cell suspension were mixed with an equal volume of phage suspension in different concentrations of $MgSO_4$ solution. After 10 min. at 37°, samples were transferred to anti-λ serum in broth so that the infected cells were able to metabolize for 20 min. before being plated with the indicator bacteria. From Table 1, it can be seen that increasing the concentration of $MgSO_4$ in the adsorption medium to 0·02 M has two marked effects—a decrease in the number of refractory bacteria and an increase in the number giving the lysogenic response. It would appear from these results that the optimal concentration of $MgSO_4$ for the adsorption of λ_{22} phage is 0·02 M since at concentrations greater or less than this, there is a progressive increase in the number of bacteria giving the 'refractory response', i.e. bacteria apparently not infected by λ phage in the conditions being studied. As the concentration of $MgSO_4$ approaches 0·02 M, not only is there an increase in the number of infected bacteria but also a change in the type of response as between lytic and lysogenic. In the presence of the lower concentrations of $MgSO_4$, the number of bacteria giving the lytic response is equal to or greater than the number giving the lysogenic response. At concentrations above 0·01 M and especially at 0·02 M, the proportion of bacteria giving the lysogenic response is much greater than that giving the lytic response. With HfrC, too, infection appears to occur more readily in the presence of 0·02 M-$MgSO_4$ than in 0·01 M-$MgSO_4$, e.g. in Table 2, when the phage input is 6·5 in 0·01 M-$MgSO_4$, 47 % of the bacteria give a refractory response, whereas in 0·02 M-$MgSO_4$, only 7 % of the bacteria are refractory. The multiplicity of phage input was greater than one in all these experiments, hence if the phage was more easily adsorbed in 0·02 M-$MgSO_4$, it is probable that in such conditions many cells became infected with more than one phage. Previous work with *Salmonella typhimurium* has shown that increasing the multiplicity of infection leads to an increased lysogenic response (Boyd, 1951).

Table 1. *Effect of concentration of* $MgSO_4$ *during adsorption of* λ_{22} *phage on the subsequent response of the cells*

In each experiment, samples of the same washed cell suspensions of *Escherichia coli* strain K112 were infected with λ_{22} in $MgSO_4$ solution: input multiplicities and final concentration of $MgSO_4$ in adsorption mixture are shown in the table. Adsorption period, 10 min. and then samples transferred to anti-λ serum in broth (1/200) and after 10 min. to broth. After a further 10 min., samples diluted in broth and plated with indicator bacteria on medium C. All incubations at 37°. Numbers in table are the mean of three plates for each sample. The viable count of the suspension was determined in each experiment. Within experimental error, the sum of the cells giving the lytic, lysogenic and refractory response equalled the initial viable count. The percentage response is expressed relative to the total number of cells giving the lytic, lysogenic and refractory response.

Expt. no.	$MgSO_4$ concn.	Multiplicity of exposure	Type of response					
			Lytic		Lysogenic		Refractory	
			No.	%	No.	%	No.	%
1	0·005	4·6	23	19	8	7	91	75
	0·01		44	44	40	40	16	16
	0·015		27	26	70	69	5	5
	0·02		17	17	73	74	9	9
2	0·005	11·0	7	8	4	5	72	87
	0·01		28	33	15	18	42	49
	0·015		18	22	54	65	11	13
	0·02		21	23	62	68	8	9
3	0·02	2·4	31	23	71	53	32	24
	0·03		24	18	44	33	67	49
	0·04		20	15	22	17	90	68
4	0·02	3·8	12	9	94	73	23	18
	0·05		7	5	24	19	97	76

Kinetics of the uptake of λ_{22} phage by Escherichia coli *strain K* 112

The uptake of λ_{22} phage by *Escherichia coli* K112 was measured in the presence of two concentrations of $MgSO_4$, 0·01 M and 0·02 M, using samples of the same cell suspension in each experiment. After mixing the pre-warmed suspensions of bacteria and phage, samples were taken at 4 min. intervals and the number of unadsorbed phage determined. The rate of uptake was faster and more prolonged in the presence of 0·02 M-$MgSO_4$ (Fig. 1A), so that in a given time more phage was adsorbed than in 0·01 M-$MgSO_4$ (92 % as compared with 52 %). Similar results were obtained with HfrC (Fig. 1B).

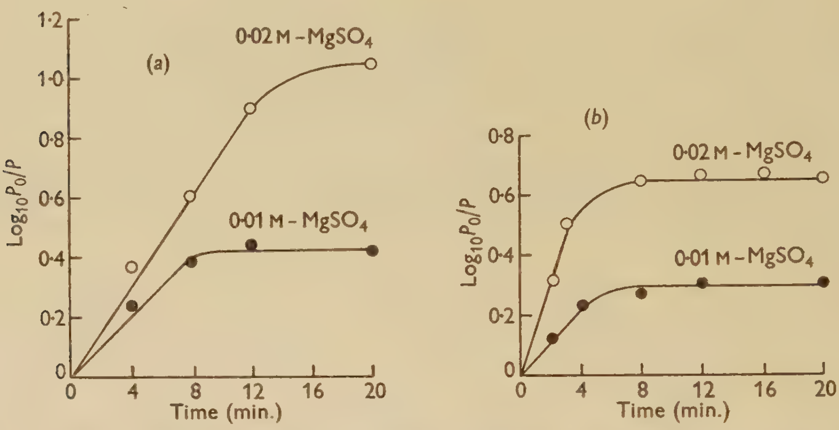

Fig. 1. Rate of adsorption of lambda phage to *Escherichia coli* strains K112 and HfrC. Washed cell suspension of *E. coli* (*a*) strain K112 ($1{\cdot}4 \times 10^8$/ml.), (*b*) strain HfrC ($2{\cdot}9 \times 10^8$/ml.) mixed at 37° with equal volume of phage suspension in $MgSO_4$, final concn. either 0·02 M or 0·01 M. Samples taken at intervals to determine unadsorbed phage. P_0 = concn. of phage at time 0; P = concn. of phage unadsorbed at time t. pH value of cells + phage = 6·65. Initial multiplicity (i.e. phage/cell) in (*a*) = 1·4; in (*b*) = 1·8.

In the foregoing experiments no attempt was made to control the hydrogen-ion concentration of the adsorption medium. It was in fact found that the pH of the mixture of cells and phage suspensions was about pH 6·5 as measured by a Beckman glass-electrode pH meter. Electrostatic forces are believed to be operative in the first stages of the attachment of phage to a bacterial cell (Puck, 1953) and since the electrical charges of the surfaces of both the bacteria and the phage will be affected by the pH value of their environment, the rates of adsorption of λ_{22} phage were determined in different conditions of hydrogen ion concentration. The adsorption media were buffered with weak potassium phosphate buffers (0·0025 M), and although the initial rates of adsorption were very similar in the pH range 5·9–7·4, the best overall adsorption (94 %) was obtained at pH 6·6 (Fig. 2). Thus, the optimal conditions for the adsorption of λ_{22} phage by *Escherichia coli* K112 are 0·02 M-$MgSO_4$ and a pH of the order 6·5.

Effect of multiplicity of infection on the lysogenic response

Samples of the same washed cell suspensions of *Escherichia coli* K112 were mixed with λ_{22} phage at four different multiplicities of input. After 10 min. at 37° samples were transferred to anti-λ serum in broth as in previous experiments, and after 20 min. at 37° the cells were plated with indicator bacteria on medium C. In addition a sample of the suspension with the lowest multiplicity of input was taken at the end of the 10 min. adsorption period and the unadsorbed phage measured. The average multiplicity of infection per cell was calculated assuming that, whatever the input multiplicity, the same proportion of phage was adsorbed and also that cells giving a refractory response did not adsorb phage. By using high multiplicities of input and thus high multiplicities of infection, it is possible to obtain populations of infected bacteria in which the number of refractory bacteria is reduced to only 1 or 2 % and the number of bacteria giving the lysogenic response is about 90 % (Table 2). Similar results were obtained with *E. coli* strain HfrC (Table 2).

Table 2. *Effect of multiplicity of infection on the type of response*

Samples of the same washed cell suspension of *Escherichia coli* strain K112 (A) or *Escherichia coli* strain HfrC (B) infected with different multiplicities of input of λ_{22} phage in 0·02 M-MgSO$_4$. Adsorption period 10 min. Samples then transferred to anti-λ serum (10 min.) and broth (10 min.) and plated on medium C with indicator bacteria. All incubations at 37°. Multiplicities of infection calculated from phage remaining unadsorbed at the end of the adsorption period in a sample from the mixture with the lowest multiplicity of input: assumed that cells giving the refractory response did not adsorb λ. (Found; in A 26 % and in B, 18 % unadsorbed phage.)

	Multiplicity of input	Multiplicity of infection	Type of response: Lytic No.	%	Lysogenic No.	%	Refractory No.	%
A	1·8	2·9	17	19	23	26	49	55
	8·8	7·1	12	13	71	78	8	9
	17·5	13	7	8	71	90	2	2
	35	26	8	9	81	90	1	1
B	1·3	1·6	22	22	42	42	37	37
	6·5	5·6	7	8	76	85	6	7
	12·9	10·9	7	8	80	89	3	3
	6·5*	3·2	27	24	34	30	53	47

* Adsorption of λ_{22} in 0·01 M-MgSO$_4$.

Effect of pretreatment with ultra-violet light before infection with λ_{22} phage

Portions (5 ml.) of the same washed cell suspensions of *Escherichia coli* K112 were irradiated with u.v. light for different periods of time, and then a sample of each irradiated suspension was diluted in broth and allowed to metabolize at 37° for 30 min. before being plated to determine the percentage survival, i.e. ability to form colonies. Other samples of the irradiated cells were mixed with λ_{22} phage in 0·02 M-MgSO$_4$ at 37° for 10 min. and then transferred to anti-λ serum and broth at 37°. After 20 min. samples were diluted and plated on medium C for the determination of type of response of the cells. In Fig. 3, it can be seen that the ability to give the lysogenic response appears to decrease more rapidly than the ability to form colonies, and these results support the idea that prior exposure to u.v. light disorganizes the bacterial nucleic acid and thus forces the phage into the lytic pathway. It is to be noted that many of the irradiated bacteria are able to give a lytic response, even though they are unable to form colonies. These results are essentially similar to those of Lieb (1953).

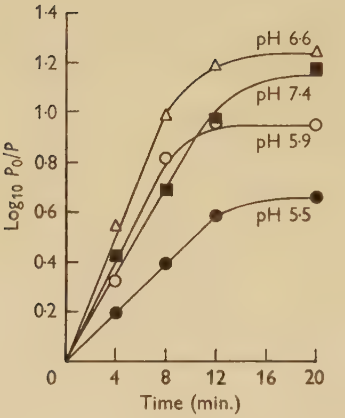

Fig. 2

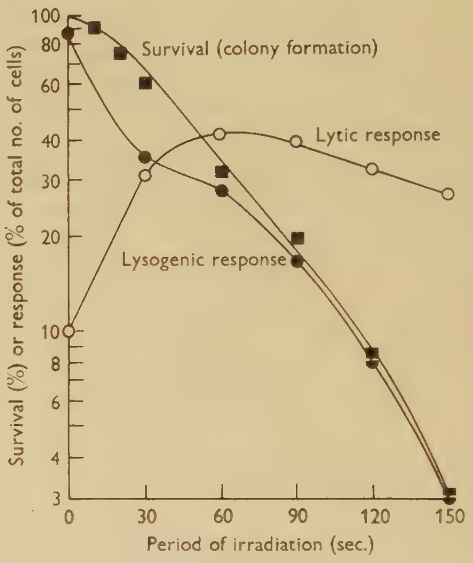

Fig. 3

Fig. 2. Effect of pH value on the adsorption of lambda phage by *Escherichia coli* strain K112. Washed cell suspension of *E. coli*. K112 ($1{\cdot}9 \times 10^8$/ml.) mixed at 37° with equal volume of phage suspension ($4{\cdot}6 \times 10^8$/ml.) in 0·005 M-phosphate buffer. Samples taken at intervals to determine phage unadsorbed. pH value of cells plus phage measured by glass electrode. P_0 = concn. of phage at time 0; P = concn. of unadsorbed phage at time t. Initial multiplicity = 2·4.

Fig. 3. Irradiation with ultraviolet light before infection and its effect on response to infection with lambda phage. Washed cell suspensions of *Escherichia coli* strain K112 ($1{\cdot}2 \times 10^8$/ml.) irradiated in thin layer in open Petri dish with ultraviolet light (lamp at 1 m.) for desired time. Samples taken and incubated in broth for 30 min. at 37°, and then plated to determine survival of ability to form colonies. Other samples mixed with equal volume of phage suspension ($1{\cdot}54 \times 10^9$), incubated at 37° for 10 min., and then samples taken to determine response of infected cells. Response expressed as % of total number of cells giving the lytic + lysogenic + refractory response in the unirradiated control.

DISCUSSION

The experiments reported here show that with λ_{22} phage and *Escherichia coli* strain K112, the optimal conditions for adsorption of the phage are 0·02 M-$MgSO_4$ at a pH value of the order 6·5. This result is in contrast with that of Kaiser (1957) who, in experiments with other λ phages, used a concentration of 0·01 M-$MgSO_4$. At the present time, it is not known why an increase in the concentration of $MgSO_4$ above 0·02 M should decrease the number of cells becoming infected (Table 1). Such a result may be due to instability of λ_{22} in the presence of high concentrations of $MgSO_4$. To date no studies have been made of the stability of λ_{22} in such conditions: indeed, although the λ phages have been used in many experiments, especially those involving genetics, there appears to be little published information concerning their basic properties.

With λ_{22} phage, it is clear that in optimal conditions (multiplicity of input about 20 and 0·02 M-$MgSO_4$) a population of infected cells can be obtained in which at least 90 % give the lysogenic response. In general, previous workers have attained no more than a 30 % lysogenic response (Kaiser, 1957), though in some experiments, up to 60 % has been reported (Lieb, 1953), but such results were not obtained consistently. Since a relatively high multiplicity of phage input is a factor influencing the number of cells which become lysogenic, it is probable that as in *Salmonella typhimurium* (Boyd, 1951), the lysogenic response is determined at least in part by the multiplicity of infection. However, the high concentration of $MgSO_4$ leading to better adsorption, and hence more phage adsorbed per cell, may not be a complete picture of the role of Mg^{++} in the establishment of lysogeny. If it can be assumed that bacteria giving the refractory response do not adsorb any phage, it can be seen from Table 2 that when the multiplicity of infection of HfrC in 0·01 M-$MgSO_4$ is 3·2, then the lysogenic response is 30 %. But though infection in the presence of 0·02 M-$MgSO_4$ at a lower multiplicity of input gave a multiplicity of infection of only 1·6, the percentage lysogenic response was 42 %. Some preliminary experiments have therefore been made to try to determine whether the concentration of Mg^{++} can directly affect the decision of the cell with regard to a lytic or lysogenic response. These experiments have not yet been completed, but it appears that during infection the main, if not the only, function of $MgSO_4$ is to be an agent for adsorption. However, if after adsorption, the concentration of $MgSO_4$ is kept at 0·02 M whilst the infected cells are metabolizing in broth, there is some evidence that the cells show a change towards the lysogenic response as compared with cells infected in the same conditions and kept for the same time in broth not supplemented with $MgSO_4$ (Fry, unpublished). Such a result could mean that Mg^{++} may directly aid the coupling of the genome of the phage to the bacterial chromosome. Alternatively, such a concentration of Mg^{++} may inhibit certain cellular processes and thus form an environment favouring the lysogenic pathway. It has been noted that *Escherichia coli* strain K112 grows more slowly in the presence of high concentrations of $MgSO_4$ and there is some evidence that reduction of metabolic activities does in fact favour lysogeny. If cells of *E. coli* strain K12S (Lieb, 1953) or *Shigella dysenteriae* (Bertani & Nice, 1954) are infected with temperate phage at 37° and then transferred to a lower temperature (20° or 25°) for an hour before plating, there is a marked change towards the lysogenic response as compared with those kept at 37° throughout the experiment. Results have also been obtained which indicate that the presence of chloramphenicol (15 μg./ml.) in the broth medium after infection of *E. coli* K112 by

λ_{22} causes a change towards lysogeny (Fry, unpublished). In such conditions, chloramphenicol is known to inhibit protein synthesis (at least 95 % inhibition) and DNA synthesis (at least 40 % inhibition) (cf. Neidhardt & Gros, 1957).

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The Metabolic Activities of *Escherichia coli* during the Establishment of Lysogeny

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SUMMARY: *Escherichia coli* strain K112 was infected with λ phage (multiplicity of input 20 particles/cell) in 0·02 M-$MgSO_4$, and 91 % infected cells gave the lysogenic response. When the infected cells were transferred to a complete amino acid medium containing ^{14}C-DL-phenylalanine and a non-utilizable inducer for β-galactosidase, the net synthesis of protein and nucleic acids did not begin until after 100–120 min. During this period there was no induced synthesis of β-galactosidase (though the enzyme was readily developed in uninfected cells) and there was no increase in turbidity of the culture or in viable cell count. The synthesis of protein and nucleic acids began in uninfected cells immediately they were placed in the complete medium, turbidity increased from the start and the viable count after 30 min. This suggested that during lysogenization the infecting phage temporarily halts most if not all of the synthetic activities of the cell and thus forms an environment in which the phage genome can become attached to the bacterial chromosome and establish the prophage state.

The biochemical changes which occur after infection in a cell which is to become lysogenic have not been studied before in any detail. Whilst much attention has been given to the biochemical effects of infection of *Escherichia coli* by the T phages, only a few experiments (cf. Lwoff, 1954–5) have been concerned with the effect of temperate phage on the metabolism of the host cell. The purpose of the present work was to attempt to study what determines the choice between the lytic and the lysogenic response, since the factors controlling this decision are not understood. At least in some bacteria, lysogeny is favoured by a high multiplicity of infection (Boyd, 1951), and after infection, changes in the environment, e.g. changes in temperature (Bertani & Nice, 1954), or the presence of substances which prevent protein synthesis (Lwoff, Kaplan & Ritz, 1954; Christensen, 1957) can alter the type of response. Conditions have been established for infecting virtually all the cells of a suspension of *E. coli* with λ phage, and thus obtaining a population in which more than 90 % of the infected cells give the lysogenic response. Some of the biochemical activities of these cells were examined and are reported here. A summary of these results has been given previously (Fry & Gros, 1958).

METHODS

The organism, *Escherichia coli* strain K112, the preparation of washed cell suspensions and stock suspension of phage lambda (λ_{22}), the composition of nutrient broth, the detection of response of infected cells and the determination of viable counts have been described previously (Fry, 1959).

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Amino acid medium (CAA) consisted of 0·2 % (w/v) casamino acids (Difco) plus 0·004 % (v/v) nutrient broth at pH 7.

Induction of β-galactosidase and estimation of activity. Iso-propylthiogalactoside (IPTG) was incorporated in medium CAA at a concn. of 10^{-3}M: in the appropriate conditions, this substance can induce the synthesis of β-galactosidase, though it is not utilized by *Escherichia coli*, even in cells in which this enzyme has been induced (cf. Monod, Cohen-Bazire & Cohn, 1951). Enzyme activity was determined directly in samples of the culture without separating the cells from the medium. A drop of toluene was added to a sample (1–2 ml.) of the culture, and the mixture incubated at 37° for 30 min. to make the enzyme (if present) accessible to the substrate (cf. Cohn & Monod, 1951). An appropriate volume (0·05–1·5 ml.) of this enzyme preparation was mixed with H_2O to 2 ml., and incubated in a standard 3 ml. Beckman cuvette at 25° with 0·5 ml. 0·02 M-*o*-nitrophenyl-galactoside (Lederberg, 1950) in 0·5 M-Na phosphate buffer, pH 7 containing M-$MgSO_4$. The increase in absorption at 420 mμ. was followed at suitable intervals of time and enzyme activity is expressed in terms of the increase in absorption at 420 mμ/ml. enzyme preparation/min.

Estimation of non-specific protein synthesis. Protein synthesis was followed in terms of the incorporation of ^{14}C from ^{14}C-DL-phenylalanine into trichloroacetic acid (TCA) precipitable material freed from nucleic acids.

Preparation of samples and determination of protein, total nucleic acid, ribonucleic acid and deoxyribonucleic acid. Duplicate samples (4 ml.) of the cultures were treated with 0·4 ml. 50 % (w/v) TCA. After standing overnight at 4°, the precipitates were collected by centrifugation at 4° and washed once with 5 % (w/v) TCA. The supernatants were rejected. Each precipitate was suspended in 0·5 ml. 5 % (w/v) TCA and heated at 100° for 30 min. in tubes capped with aluminium foil. The residue was collected by centrifugation and the supernatant separated carefully and kept for the determination of material absorbing at 260 mμ (total nucleic acid), ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). The residue was washed with 1 ml. 60 % (v/v) ethanol and then dissolved in 0·5 ml. NH_4OH (1 part sp.gr. 880; 2 parts H_2O). Samples (0·2 ml.) of the ammoniacal solution were spread and dried on metal planchets for the counting of ^{14}C at infinite thinness using a Geiger–Muller tube with an end window. Total nucleic acid was determined in terms of absorption at 260 mμ in samples of the hot TCA extract (0·3 ml. extract from the cells of 4 ml. of the culture was diluted to 3 ml.: the blank cell contained 0·5 % (w/v) TCA). For the estimation of DNA, samples of the hot TCA extract were diluted to 1 ml. and mixed with 0·2 ml. 3 N-perchloric acid and 1·2 ml. Dische reagent (Burton, 1956). After 4 hr. at 37° in a water bath, the absorption of the violet colour was measured at 590 mμ. A standard was prepared from a sample of sodium thymonucleate (Pointet & Girard, Paris) previously heated with 5 % (w/v) TCA and taken through the same procedure. For the estimation of RNA, 0·4 ml. of the hot TCA extract was mixed with 1·1 ml. H_2O, 1·5 ml. $FeCl_3$ (0·1 %, w/v) in concn. HCl and 0·15 ml. orcinol (10 %, w/v) in ethanol, and the mixture heated at 100° for 40 min. (Schneider, 1945). After cooling, the absorption of the green colour was determined at 650 mμ. The standard was sodium nucleate (Pointet & Girard, Paris).

Measurements of turbidity of cultures and suspensions were made in a Meunier Spectrophotometer using the red filter. Where required, cultures were diluted until the cell concentration was in the range such that the apparatus gave a linear response to changes in turbidity.

Spectrophotometry. A Beckman DU Universal Spectrophotometer was used and cells with a light path of 1 cm.

Experimental procedure

A culture of *Escherichia coli* strain K112 was grown in nutrient broth and harvested when it was about two-thirds of the way through the logarithmic phase of growth. A washed cell suspension (2×10^8 cells/ml.) was prepared, divided into two parts and warmed at 37°. One part was mixed with an equal prewarmed volume of λ phage in 0·04 M-$MgSO_4$ (4×10^9 λ/ml.), giving a multiplicity of exposure of 20λ/cell. The other part, the uninfected control cells, was mixed with an equal volume of prewarmed 0·04 M-$MgSO_4$ alone. After 10 min. at 37°, the cells were separated by centrifugation and resuspended in prewarmed medium CAA (65 ml.) containing ^{14}C-DL-phenylalanine (2×10^{-4} M, 20,000 c.p.m./μmole) and IPTG (10^{-3} M), the non-utilizable inducer for β-galactosidase. The two cultures of infected and uninfected cells were incubated at 37° in 500 ml. conical flasks shaking gently in a water bath. Samples were taken at intervals for the determination of: (i) growth in terms of turbidity and viable cell count; (ii) protein synthesis in terms of the induced synthesis of β-galactosidase and the incorporation of ^{14}C into cell protein, and (iii) total nucleic acid, DNA and RNA by the analysis of hot TCA extracts of the cells. Furthermore, after the infected cells had been incubated for 10 min., a sample was transferred to anti-λ serum in broth and then plated in triplicate for the detection of the response of the infected cells (Fry, 1959). None of the cells were refractory, 9 % gave the lytic response and 91 % gave the lysogenic response, thus showing that the majority of the infected cells were cells in which lysogenization was to take place.

RESULTS

After being transferred to the casamino acid medium (CAA), the uninfected cells began to grow, and whilst turbidity increased logarithmically from the start, there was a lag of about 30 min. before an increase in the viable cell count was detected, and after about 45 min. the viable cell count was also increasing logarithmically (Fig. 1). These results are to be contrasted with those of the culture of infected cells, in which turbidity did not begin to increase until after 100 min. and the viable count had just begun to increase after 145 min. incubation (Fig. 1). Since most (91 %) of the infected cells were shown to give the lysogenic response, these results indicate that at least one of the metabolic activities which contribute to the synthesis of cell material is temporarily halted in cells in which the process of lysogenization is taking place and that this is revealed as a lag in the growth of a culture of these cells. The formation of β-galactosidase was not induced to any significant extent throughout the period of 190 min. after infection (Fig. 2). In later experiments, with longer

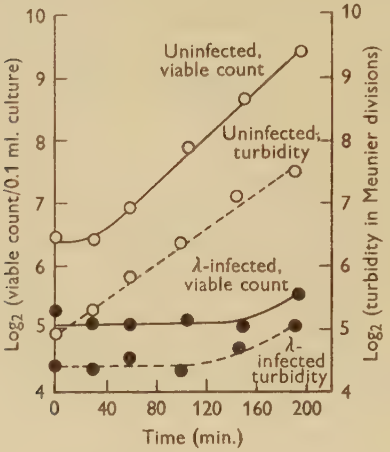

Fig. 1

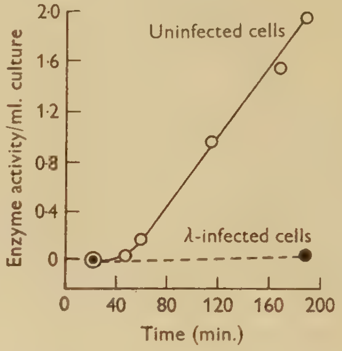

Fig. 2

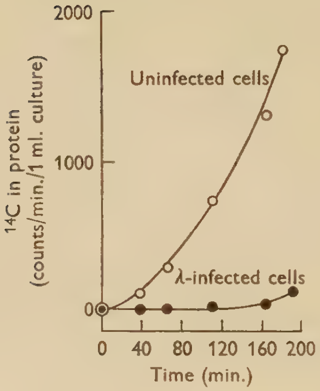

Fig. 3

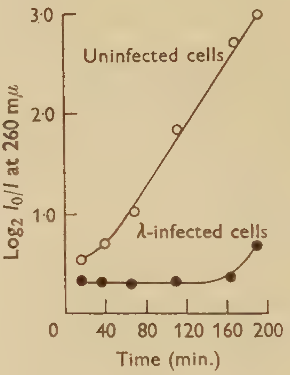

Fig. 4

Fig. 1. Growth of uninfected and λ-infected *Escherichia coli* strain K112. Cultures of uninfected and of λ-infected cells in medium CAA ($+$IPTG$+^{14}$C-phenylalanine) incubated at 37° and samples taken at intervals for determination of viable count and turbidity. In this and all other figures, time refers to period of incubation in medium CAA.

Fig. 2. Induction of β-galactosidase by *iso*propylthiogalactoside (IPTG) in uninfected and λ-infected *Escherichia coli* strain K112. β-galactosidase activity determined by the hydrolysis of *o*-nitrophenylgalactoside. Enzyme activity expressed as increase in absorption (log I_0/I) at 420 mμ/ml. of culture/min.

Fig. 3. Incorporation of ^{14}C from ^{14}C-phenylalanine into the protein of uninfected and λ-infected *Escherichia coli* strain K112. After cells extracted with hot TCA, the residues were collected, washed with ethanol and dissolved in NH_4OH. Samples of the ammoniacal solution dried on metal planchets for determination of ^{14}C.

Fig. 4. Synthesis of material absorbing at 260 mμ (nucleic acids) in λ-infected and uninfected *Escherichia coli* strain K112. Hot TCA extracts of cells prepared and absorption measured at 260 mμ. Extracts diluted when required and absorption value multiplied by appropriate dilution factor.

incubation periods, it was found that β-galactosidase activity was developed once the culture was growing at a rate comparable with that of the uninfected cells. In the culture of uninfected cells, the induction of β-galactosidase took place readily after the cells had been in the growth medium for about 40 min. The lag in the induction in the uninfected cells is probably to be accounted for in terms of the concentration of amino acids in the medium being high enough to cause some initial inhibition of the induction process (cf. Cohn & Monod, 1953). It would therefore appear that the protein synthesis is at least very much reduced in the λ-infected cells, and this conclusion is supported by the finding that no incorporation of ^{14}C into the protein of these cells was detected until after 120 min. incubation in the growth medium (Fig. 3). However, in the uninfected cells, the incorporation of ^{14}C into protein followed the course to be expected in a culture growing logarithmically (Fig. 3). Examination of the hot TCA extracts of the infected cells for total nucleic acid, measured in terms of absorption at 260 mμ., showed that there was no detectable increase in nucleic acid until after 140 min., whereas the total nucleic acid content of the uninfected cells increased very rapidly (Fig. 4). Determinations of the separate nucleic acids showed that there was no apparent net synthesis of RNA or DNA in the infected cells until after 120–140 min. incubation, but in the uninfected cells, DNA and RNA synthesis proceeded logarithmically from the start (Fig. 5).

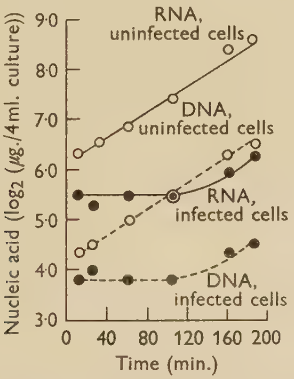

Fig. 5. Synthesis of RNA and DNA in uninfected and λ-infected cells of *Escherichia coli* strain K112. Hot TCA extracts of cells assayed for DNA and RNA (see Methods, p. 686).

DISCUSSION

From the results of the experiment described here, it appears that immediately after infection there is a profound disturbance in the metabolic activities of those cells in which the prophage of λ is being established, and that the net synthesis of protein and nucleic acid is blocked for an appreciable time, and, although the cell remains viable, there is inevitably a delay in cell division. Previous workers (Bertani & Nice, 1954; Lieb, 1953) have also noted this delay in division in cells giving the lysogenic response. Since the test for the response of the infected cells showed that 9% gave the lytic response, it may be asked why no synthesis of protein or nucleic acid was detected in the first 45 min. (the normal latent period for λ) when the infected cells were incubated in the amino-acid medium. It is in fact possible that all the cells gave the lysogenic response when transferred to the liquid medium and that the response of a small proportion was changed towards the lytic response when they were plated on solid media 20 min. after infection. It is well known that environmental changes can greatly affect the type of response to be elicited from infected cells (cf. Bertani, 1958), and it is feasible that manipulations involved in plating the bacteria in the response test may bring about such changes. Even if there was a small percentage of cells in which phage was maturing, it is possible that the experimental techniques were not sensitive enough to detect net synthesis on such a small scale. For example, DNA synthesis was not detected in *Escherichia coli* infected with T_7, although the phage undoubtedly went into the vegetative state and multiplied (Kozloff, 1953). Whilst the possibility of some metabolic activity has not been excluded, the conclusion can be drawn from the results of the experiment reported here that the entry of phage material into the host cell either directly or indirectly blocks at least the majority of the synthetic reactions in those cells in which lysogenization is taking place. Though this belief is naïve, it is not possible at the present time to elaborate it in detail and incorporate all the available experimental results into an unequivocal statement of what is happening during lysogenization.

Bertani & Nice (1954) have found that *Shigella dysenteriae* can be turned from the lytic response towards lysogeny by taking P1-infected cells in complete medium at 37° and placing them at a lower temperature (24°) for 2 hr.; this effect can be produced even at a comparatively late stage (20 min.) in the latent period (Bertani, 1953). With the *Escherichia coli* strain K12-λ system studied by Lieb (1953), though raising the temperature from 37° to 46° appeared to promote the lytic rather than the lysogenic response, decreasing the temperature did not favour lysogeny. Stent & Fuerst (1956), using λ phage labelled with ^{32}P, found only minor difference in the stabilization to decay of ^{32}P in cells of *E. coli* giving the lytic response as compared with those giving the lysogenic response. Within 30 min. after infection, the genome of the phage in cells being lysogenized was in a state such that it was no longer susceptible to the decay of ^{32}P. Stent & Fuerst suggested that for a period immediately after infection, there was probably a common pathway for lysogeny and vegetative phage, and they deduced that little, if any, of the DNA of the invading phage becomes joined to the bacterial nuclear material to form the prophage. There is thus some indirect evidence that after infection the initial steps are the same irrespective of whether the cell is to give a lysogenic or a lytic response, and that an irrevocable decision as to the type of response is not made until some minutes after the infected cells have been placed in a complete medium. However, in bacteria infected with temperate phage and giving the lytic response the synthesis of protein and RNA still proceeds at rates comparable with those prior to infection, though DNA synthesis is stopped for several minutes (cf. Lwoff, 1954-5). But in the experiment reported here, dealing with cells undergoing lysogenization, there were no obvious signs of protein or RNA synthesis, indicating that relatively soon after infection the process of lysogenization is different from that leading to vegetative growth and the production of infective phage particles. It is well known from experiments with *E. coli* and the T phages that DNA and RNA synthesis is stopped immediately after infection, and that protein synthesis is restricted. Only after a certain amount of protein synthesis has occurred can DNA synthesis be resumed. Whether analogies should be sought between the effect of the T phages and of λ phage on the metabolism of *E. coli* is, however, doubtful, since there appears to be at least one fundamental difference between these two types of virus. The phage coats of the T viruses can themselves kill cells (Herriott, 1951; French & Siminovitch, 1955), whereas such a lethal effect has not been demonstrated with phage coats of either wild type or virulent mutants of λ (Weigle, quoted by Bertani, 1958).

In view of the striking reduction in the metabolic activities of cells during lysogenization, it is interesting to note that conditions which slow or inhibit metabolism, e.g. a reduction in temperature (Bertani & Nice, 1954) or the presence of chloramphenicol (Christensen, 1957), appear to favour lysogeny. The significance of a high multiplicity of infection in lysogeny is not understood, but there is a suggestion that each phage particle can contribute something to inhibit the biochemical activity of the host cell and that if the multiplicity of infection is sufficiently high, these inhibitory effects are additive (cf. Hershey, 1955, 1957) and the activity of the host may thus be reduced so much that the end result is an environment in which the phage genome can become attached to the bacterial chromosome and not enter the vegetative phase of phage multiplication. If such a suggestion is correct, then it is feasible that if a cell is infected with only one particle, any artificial means of retarding the metabolic activity of the cell will also favour lysogenization. Evidence that the phage itself as well as the physiological condition of the host cell has something to contribute to the decision of lysogeny or phage production comes from the work of Levine (1955). Phage mutants which cannot themselves bring about lysogeny, can be brought into the prophage state if the cell is simultaneously infected with mutants of another but related type (which also by themselves are not capable of entering the prophage state). In other words, mixed infection led to lysogeny for what is usually called a 'virulent' phage, that is one capable of giving only the lytic response.

During the lag in the growth of the cells giving the lysogenic response, it is probable that the prophage is becoming established in the bacterial chromosome, and in order to determine how soon after infection this has been accomplished, various techniques can be exploited, e.g. sensitivity to ultraviolet light, the onset of immunity to infection by mutants of λ and T phages, and the ability to take part in bacterial recombination with the consequence of zygotic induction. It is hoped that the result of such experiments will contribute to an understanding of the process of lysogenization.

This work was undertaken whilst one of us (B.A.F.) held a French Government Exchange Fellowship. We wish to express our sincere appreciation of the help and advice which we received from Dr A. Lwoff and Dr F. Jacob.

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The Influence of Certain Derivatives of Vitamin B_{12} upon the Growth of Micro-Organisms

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SUMMARY: Seven analogues of vitamin B_{12} were examined for their influence upon the growth of *Ochromonas malhamensis*, *Euglena gracilis*, *Lactobacillus leichmannii* ATCC 4797 and *Escherichia coli* 113–3. The analogues were active in promoting the growth of *E. gracilis* and *L. leichmannii*, but were essentially inactive for *O. malhamensis*. For *E. coli* three of the analogues were active and four inactive. In *O. malhamensis* and in *E. coli*, the 'inactive' analogues antagonized the growth-promoting action of cyanocobalamin, apparently by saturating the cells' mechanism for 'binding' the vitamin and so preventing its uptake.

Tests were also carried out with *Ochromonas malhamensis* and *Escherichia coli* on twelve derivatives of benziminazole. None of these proved inhibitory towards *E. coli*, at least up to 100 μg./ml. culture medium. Eight of the compounds were inhibitory towards *O. malhamensis*; the effects were relatively small, however, and were possibly unrelated to the metabolism of vitamin B_{12}.

At least fourteen analogues of vitamin B_{12} occur in nature. They are all closely related chemically but differ in their growth-promoting activities for different micro-organisms. Of the analogues so far tested, only those few that contain a benziminazole moiety in their nucleotide have proved active in promoting the growth of higher animals. The remainder are inactive, and even in some instances have been shown to antagonize the action of vitamin B_{12} (Coates *et al.* 1956). Certain micro-organisms, among them the protozoan *Ochromonas malhamensis*, share this animal pattern of response to the B_{12}-vitamins. Other micro-organisms, including the familiar assay organisms *Lactobacillus leichmannii*, *Euglena gracilis* and *Escherichia coli* 113-3, are less exacting and can make equivalent use of several of the B_{12}-group vitamins (cf. Coates & Ford, 1955).

Tests with *Ochromonas malhamensis* (Ford, 1958) and with a group of soil bacteria (Ford & Hutner, 1957), for which the analogues, pseudovitamin B_{12} and Factor A, are essentially inactive, revealed a form of metabolite-antimetabolite relationship between cyanocobalamin and each of these 'inactive' analogues. The analogues inhibited growth apparently by saturating the cells' mechanism for trapping and storing cyanocobalamin. The process of absorption required the presence of an intact nucleotide in the molecule of the B_{12}-vitamin. Factor B, which constitutes the non-nucleotide portion of all the B_{12}-vitamins that have so far been characterized, was not absorbed and was neither active nor antagonistic.

The present paper reports an extension of these earlier studies. Through the kindness—deeply appreciated— of Professor K. Bernhauer (Biochemisches Laboratorium der Aschaffenburger Zellstoffwerke, A.G., Stokstadt-am-Main) and Dr E. L. Smith, F.R.S. (Glaxo Laboratories Ltd., Greenford, Middlesex) I have been able to examine in a number of micro-organisms the effects on growth and cyanocobalamin uptake of several derivatives of cyanocobalamin, prepared by chemical modifications of the structures of naturally occurring forms of the vitamin. A more limited study has also been made of the effects of various benziminazole derivatives on the growth of *Ochromonas malhamensis* and of *Escherichia coli*. It was prompted by reports (cf. Funk & Nathan, 1958) that various substituted benziminazoles inhibit the growth of bacteria and flagellates that require vitamin B_{12}.

MATERIALS

Analogues of vitamin B_{12}

Pseudovitamin B_{12}, vitamin B_{12} monocarboxylic acids, vitamin B_{12} methylamide, vitamin B_{12} ethylamide, vitamin B_{12} anilide (all made by Dr E. Lester Smith; described by Smith, Parker & Gant, 1956), 'benzyl $B_{12\text{III}}$' (an analogue of cyanocobalamin, containing in its nucleotide the benzyl ether of 5-hydroxybenziminazole, made by Professor K. Bernhauer; described by Gross, Friedrich & Bernhauer, 1957) and 'MMHP' (an analogue of pseudovitamin B_{12}, containing 2-methyl-mercapto-6-hydroxypurine in its nucleotide, made by Professor K. Bernhauer; described by Friedrich & Bernhauer, 1957) were studied.

Derivatives of benziminazole

Benziminazole, 5-methylbenziminazole, 5-aminobenziminazole, 5-nitrobenziminazole, 2-(*o*-hydroxyphenyl)benziminazole, 5:6-dimethylbenziminazole, 5:6-dichlorobenziminazole, 2:5-dimethylbenziminazole, 3:4-dimethyl-6-D-ribitylaminobenzene, 1-amino-3:4-dimethyl-6-D-ribitylaminobenzene, 5:6-dimethylbenziminazole-α-D-ribofuranose phosphate (α-ribazole), 5:6-dimethylbenziminazole-β-D-ribofuranose phosphate (β-ribazole) were studied.

METHODS

Microbiological methods

For the tests with *Euglena gracilis* the 'Z' strain of this organism was used in the assay procedure recommended by Hutner, Bach & Ross (1956).

The tests with *Lactobacillus leichmannii* ATCC 4797 were carried out by the method of Skeggs, Nepple, Valentik, Huff & Wright (1950), modified as described by Coates, Ford, Harrison, Kon & Porter (1953). With *Escherichia coli* 113–3 a tube assay technique was used essentially as described by Burkholder (1951). The basal medium was modified by the inclusion of sodium cyanide (2 mg./l. final strength medium) and the substitution of thiomalic acid for thioglycollic acid. For comparing the effects of the vitamin B_{12} analogues on the growth rate, a heavy inoculum of exponentially growing cells was given, and the period of incubation was reduced to 10 hr. Cyanobalamin was included in the basal medium at a growth-limiting level, and the various analogues were tested over a wide range of concentrations.

Assays with *Ochromonas malhamensis* were done as described by Ford (1958). An examination of the influence on growth rate exerted by the different analogues of vitamin B_{12}, and by the various derivatives of benziminazole, was carried out as described by Ford (1958). So also were the studies on vitamin B_{12} uptake by resting cells, except that in the experiments now described ^{58}Co-labelled cyanocobalamin was employed, and measured with a scintillation counter containing a thallium-activated sodium iodide crystal.

An extract of *Ochromonas malhamensis* cells was prepared in the following manner. Two litres of 4-day culture grown with 0·0001 μg. cyanocobalamin/ml. were centrifuged, and the supernatant fluid was discarded. The residue was reconstituted to a thick brei by the addition of 25 ml. of 0·3 M-sucrose solution and transferred to a 150 ml. conical flask, together with 30 ml. of 3 mm. glass beads. The flask was stoppered tightly and shaken vigorously for 20 min. in a Towers-Gilson vibratory shaker (Towers Ltd., Widnes, England). The contents of the flask were then filtered through a plug of glass wool in the stem of a small funnel, and centrifuged at 10,000 *g* for 20 min. The supernatant liquor was carefully decanted for use in the experiment described on p. 698.

The technique of ultrafiltration through cellulose sausage skin has been described by Gregory (1954).

RESULTS

Measurements of the growth-promoting activities of the vitamin B_{12} *analogues*

The relative growth-promoting activities of the vitamin B_{12}-analogues for the four different test micro-organisms are shown in Table 1. None of the seven compounds was active for *Ochromonas malhamensis*; three were active for *Escherichia coli*, and all were active for *Lactobacillus leichmannii* and *Euglena gracilis*.

Table 1. *Relative growth-promoting activities of different analogues of vitamin B_{12}, measured with different micro-organisms. The activity of cyanocobalamin is taken as* 100

	Ochromonas malhamensis	*Escherichia coli*	*Lactobacillus leichmanii*	*Euglena gracilis*
Vitamin B_{12} methylamide	0	0	14	26
Vitamin B_{12} ethylamide	0	0	12	13
Vitamin B_{12} anilide	0	0	48	7
Benzyl-vitamin $B_{12\,\mathrm{III}}$	0	15–45	36	30
Vitamin B_{12} monoacids	0	0	23	184
Pseudovitamin B_{12}	0	8	62	70
‘MMHP’	0	43	25	Not tested

Influence of the analogues upon the growth-rate of Ochromonas malhamensis *and* Escherichia coli

Six of the vitamin B_{12}-analogues were tested for their effects upon the growth responses of *Ochromonas malhamensis* and *Escherichia coli* to cyanocobalamin. The results of these tests are illustrated in Figs. 1 and 2, which show approximately the rates at which cultures of the organisms grew in presence of a constant level of cyanocobalamin and with graded concentrations of the various analogues. Under the conditions of test employed, growth rates were nearly proportional to the logarithms of the final optical densities of the cultures. The ratios, concentration of vitamin B_{12} analogue: concentration of cyanocobalamin, at which the rates of growth were reduced to one half that of the culture grown with cyanocobalamin alone, were taken as an index of the antagonistic action of the analogues. These ratios are given in Table 2.

Ochromonas malhamensis and *Escherichia coli* were alike in that, for both organisms, vitamin B_{12} methylamide and vitamin B_{12} ethylamide were potent antagonists, and the 'anilide' markedly less antagonistic. The monoacids were strongly active against *E. coli* and only weakly inhibitory towards *O. malhamensis*. Benzyl $B_{12\,\mathrm{III}}$ and MMHP, on the other hand, promoted the growth of *E. coli* but inhibited the growth of *O. malhamensis*. In this behaviour

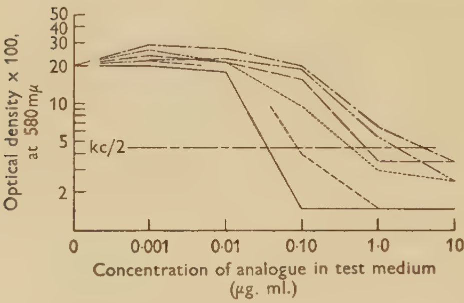

Fig. 1

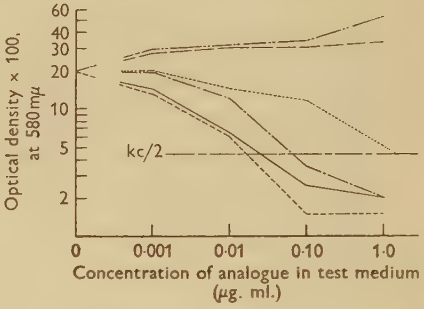

Fig. 2

Fig. 1. Influence of vitamin B_{12} analogues on the growth of *Ochromonas malhamensis* in medium containing 0·15 mμg. cyanocobalamin/ml.

Fig. 2. Influence of vitamin B_{12} analogues on the growth of *Escherichia coli* 113-3 in medium containing 0·2 mμg. cyanocobalamin/ml.

———, vitamin B_{12} methylamide; ·········, vitamin B_{12} ethylamide; – – –, vitamin B_{12} anilide; — · — ·, vitamin B_{12} monacids; —–—, Benzyl vitamin $B_{12\,\mathrm{III}}$; — · · —, 'MMHP'.

In both figures, optical densities are plotted on a logarithmic scale, so that the curves indicate approximately the rates of cell division at the different concentrations of the analogues. The line kc/2 shows the concentrations of the different analogues at which the growth-rate was about half that in the control cultures.

Table 2. *Concentrations of vitamin B_{12}-analogues, relative to cyanocobalamin, at which growth-rates were reduced to half those of control cultures grown with cyanocobalamin alone.*

	Relative concentration of analogue required to reduce growth rate by 50 %	
	Ochromonas malhamensis test	*Escherichia coli* test
Vitamin B_{12} methylamide	240	126
Vitamin B_{12} ethylamide	580	83
Vitamin B_{12} anilide	2,800	6,400
Benzyl-vitamin $B_{12\,\mathrm{III}}$	4,500	Not antagonistic
Vitamin B_{12} monoacids	26,000	330
'MMHP'	12,000	Not antagonistic

Escherichia coli was grown in basal medium containing 0·2 mμg. cyanocobalamin/ml. and *Ochromonas malhamensis* in medium containing 0·15 mμg. cyanocobalamin/ml.

they resembled the naturally occurring analogues pseudovitamin B_{12} and Factor A (Ford, 1958).

Influence of benziminazole derivatives upon the growth-rate of Ochromonas malhamensis *and* Escherichia coli

The analogues listed on p. 694 were tested at concentrations of 0·01, 0·10, 1·0, 10 and 100 μg./ml. for their effects upon the rate of growth of *Ochromonas malhamensis* and *Escherichia coli* in culture media containing 0·1 mμg. cyanocobalamin/ml. None of the compounds depressed the growth of *E. coli.* At 100 μg./ml., 5-methylbenziminazole and 2:5-dimethylbenziminazole were slightly stimulatory.

Benziminazole, 5-methylbenziminazole, 5-nitrobenziminazole, 5:6-dimethylbenziminazole, 5:6-dichlorobenziminazole and 2:5-dimethylbenziminazole were slightly inhibitory towards *Ochromonas malhamensis* at 10 μg./ml. and 2-(*o*-hydroxyphenyl) benziminazole caused a marked depression of growth. At 100 μg./ml. the effects of the different analogues on relative growth rate were more pronounced, as follows: growth-rate in absence of benziminazole analogue, 100; with 5-aminobenziminazole, 100; with α-ribazole, 98; with 3:4-dimethyl-6-D-ribityl-aminobenzene, 95; with β-ribazole, 93; with 1-amino-3:4-dimethyl-6-D-ribityl-aminobenzene, 91; with 2:5-dimethylbenziminazole, 88; with benziminazole, 75; with 6-nitrobenziminazole, 73; with 5-methylbenziminazole, 64; with 5:6-dimethylbenziminazole, 27; with 5:6-dichlorobenziminazole, 16, and with 2-(*o*-hydroxyphenyl)benziminazole, 8.

Influence of vitamin B_{12} analogues and benziminazole derivatives upon the uptake by Ochromonas malhamensis *of ^{58}Co-labelled cyanocobalamin*

Forty-five conical flasks of 100 ml. capacity were arranged and numbered into nine series of five. To each flask was added 0·026 μg. (0·05 μc.) of ^{58}Co-labelled cyanocobalamin, together with 0, 0·05, 0·50, 5·0 or 50 μg. of one of nine compounds (seven vitamin B_{12}-analogues, 5:6-dimethylbenziminazole and 2-(*o*-hydroxyphenyl)benziminazole). Next was added 30 ml. of *Ochromonas malhamensis* culture, from 2 l. culture grown for 4 days in light with 0·1 mμg. cyanocobalamin/ml., as described by Ford (1958). The flasks and their contents were stood for 1 hr. at room temperature (*c.* 26°), being shaken at 10 min. intervals to prevent sedimentation of the organisms in the culture. The flask contents were then centrifuged, the supernatant liquors were decanted and the residua of compacted cells dissolved in 10 ml. portions of 4N-NaOH solution. The radioactivities of these solutions were then measured. The findings are shown in Fig. 3. The seven analogues of vitamin B_{12} were alike in that at the higher test concentrations they prevented the uptake of cyanocobalamin, whereas at lower concentrations they facilitated uptake. The effects of the benziminazole derivatives were relatively small. The lowest test concentration of 2-(*o*-hydroxyphenyl)benziminazole enhanced slightly the uptake of cyanocobalamin; the highest concentration was slightly inhibitory. 5:6-Dimethylbenziminazole caused a small increase in cyanocobalamin uptake at all but the lowest test concentration.

Influence of vitamin B_{12}*-methylamide upon the 'binding' of cyanocobalamin by an extract of* Ochromonas malhamensis *cells*

To each of four test tubes was added 0·026 μg. (0·05 μc.) ^{58}Co-labelled cyanocobalamin, together with 0, 0·5, 5·0, or 50 μg. vitamin B_{12}-methylamide. Water was added to bring the content of each tube to 7 ml., followed by an extract of *Ochromonas malhamensis* cells to 10 ml. The whole was then ultrafiltered until about 3·5 ml. filtrate had collected. A 3 ml. portion of each filtrate was taken

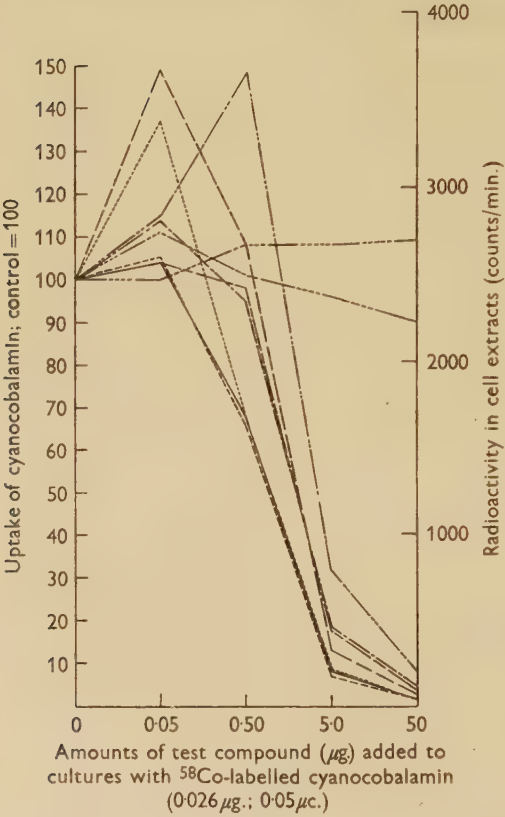

Fig. 3

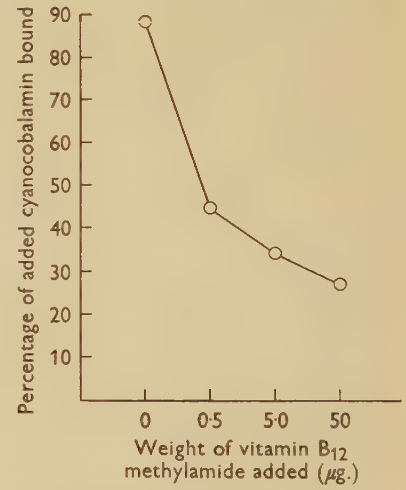

Fig. 4

Fig. 3. Effects of vitamin B_{12} analogues and of benziminazole derivatives on the uptake of cyanocobalamin. ^{58}Co-labelled cyanocobalamin (0·026 μg.; 0·05 μc.) was added to 30 ml. portions of a culture of *Ochromonas malhamensis*, together with graded amounts of the test compound. After incubation for 1 hr., the cells were separated from culture fluid and dissolved in 4 N-NaOH for scintillation counting. ———, vitamin B_{12} methylamide; – –, vitamin B_{12} ethylamide; – – – –, vitamin B_{12} anilide; — –, vitamin B_{12} monacids; ————, benzyl vitamin $B_{12\text{III}}$; ——- - ——, 'MMHP'; ——— — ———, Pseudovitamin B_{12}; — - - - - —, 2-(*o*-hydroxyphenyl)benziminazole; ——- - - - - ——, 5:6-dimethylbenziminazole.

Fig. 4. Influence of vitamin B_{12}-methylamide on the binding of cyanocobalamin. ^{58}Co-labelled cyanocobalamin (0·026 μg.; 0·05 μc.) was added to equal portions of an extract of *O. malhamensis* cells, together with graded amounts of vitamin B_{12}-methylamide. Each was then ultrafiltered, and the radioactivity of the filtrates measured.

and diluted to 10 ml. Finally, the radioactivities of these diluted filtrates were measured. The results are shown in Fig. 4. They indicate that the binding of added cyanocobalamin by an extract of *O. malhamensis* cells was progressively reduced by the simultaneous addition of progressively increasing amounts of vitamin B_{12}-methylamide.

Analysis of *Ochromonas malhamensis* cells grown with limiting cyanocobalamin showed that the soluble cell proteins contained only 10 % of the cells' nitrogen, but nearly 30 % of the cyanocobalamin, in a non-ultrafiltrable form. The experiment was therefore repeated, but substituting a particle-free cell preparation obtained by centrifuging a cell extract at 100,000 *g* for 20 min. The results showed that this preparation also had the property of binding added cyanocobalamin, and again this binding was prevented by the simultaneous addition of vitamin B_{12}-methylamide.

DISCUSSION

All of the vitamin B_{12}-analogues tested were active for *Euglena gracilis* and *Lactobacillus leichmannii.* It remains to be investigated whether the compounds are intrinsically active, or whether these two micro-organisms can bring about their conversion to cyanocobalamin.

It has been shown (Ford, 1958) that pseudovitamin B_{12} is taken up by *Ochromonas malhamensis* in about the same amount as cyanocobalamin. The inactivity of the analogue as a growth promoter cannot be attributed to inefficient uptake; the compound is inherently inactive. The process of vitamin B_{12}-uptake is associated with the presence in the organism of a specific vitamin B_{12}-binding component, which has the property of 'binding' cyanocobalamin and pseudovitamin B_{12} in the sense of preventing their passage through a cellophane membrane. Cyanocobalamin is bound preferentially when it is added to a cell extract in admixture with pseudovitamin B_{12}. However, in relatively high concentration, pseudovitamin B_{12} prevents the uptake of cyanocobalamin and so inhibits growth.

There remains the paradox that, at lower concentrations, pseudovitamin B_{12} facilitates the uptake of cyanocobalamin and potentiates its growth-promoting activity (Ford, 1958). This is explained by the observation that, in cultures of *Ochromonas malhamensis*, the vitamin B_{12}-binding factor is not confined to the organisms: it appears also in the culture fluid during growth, and combines there with free vitamin B_{12}. In this complex-bound form the vitamin is far less readily absorbed than it is in its free form. One may suppose that, at relatively low concentration, pseudovitamin B_{12} competes with cobalamin for the B_{12}-sequestering substance in the culture fluid, and so releases free cobalamin to the organisms. At higher concentrations the analogue tends increasingly to inhibit growth by blocking the intracellular binding mechanism.

It seems highly probable that all the B_{12}-analogues tested in the present study act on growth and vitamin B_{12}-uptake in *Ochromonas malhamensis* in this same fashion. Such quantitative differences as are apparent (Figs. 1, 3) can be explained in terms of the relative affinities of analogue and vitamin for the binding substance.

The effects of the various B_{12}-analogues upon growth in *Ochromonas malhamensis* are shown most sensitively during the period of exponential growth, when the rate of cell division is governed by the concentration of available vitamin B_{12} (Ford, 1958). Prolonging the period of incubation gives a different picture, that may be complicated by the effects of exhaustion of nutrients in the culture medium or by adaptation of the organism to the use of the analogue. Experiments now in progress suggest that *O. malhamensis* adapts fairly readily to the utilization of B_{12}-methylamide, or more probably that vigorously growing cultures can slowly transform this analogue to vitamin B_{12}. Thus the methylamide may be judged 'strongly inhibitory' at 48 hr., and yet appear at 96 hr. to potentiate the growth-promoting effect of vitamin B_{12}. Similarly, with mixtures of vitamin B_{12} and pseudovitamin B_{12}, the amount of growth finally achieved is determined largely by the amount of vitamin B_{12} present, and is little affected by the analogue. All this underlines the necessity, when reporting studies of this nature, to specify in some detail the conditions under which the tests were carried out.

None of the benziminazole derivatives was strongly inhibitory. 2-(*o*-hydroxyphenyl)benziminazole was the most active, and comparison of the response curves in Fig. 3 suggests that its mode of action might well be the same as that of the vitamin B_{12}-analogues. The effects were relatively slight, however, and were quite possibly non-specific.

In a recent publication, Baker, Pasher, Hutner, Herbert & Sobotka (1959) have reported that the methylamide, ethylamide and anilide of the monocarboxylic acid of vitamin B_{12} were active in promoting the growth of *Euglena gracilis* and *Lactobacillus leichmannii*, and inactive for *Ochromonas malhamensis* and *Escherichia coli*. They pronounced that the three compounds were 'inactive as B_{12}-antagonists despite their previous designation as "antivitamin B_{12}" substances'. This conclusion should have been qualified by a statement that the concentrations of analogues used in the tests were relatively low, $< 0{\cdot}001$ μg./ml. (S. H. Hutner, private communication). The authors had not enough of the analogues to enable them to try the higher concentrations tested in the present study. It can be seen from Fig. 1 that at the concentration of 0·001 μg./ml.—and indeed at 0·01 μg./ml.—no one of these three analogues inhibited the growth of *O. malhamensis*. Similarly, at 0·001 μg./ml. the analogues had no large effect upon the growth of *E. coli*.

I wish to thank Dr S. K. Kon for his helpful interest in this work. I am grateful also to Mr S. H. Phillips of the department of Radiobiochemistry for his help, in measuring the radioactivities of numerous test preparations.

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The Bulk Growth of Animal Cells in Continuous Suspension Culture

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SUMMARY: Several methods for growing animal cells in suspension culture were examined, to find the most efficient in terms of cells produced in a given time for the minimum of medium and attention. Continuous medium flow was more efficient than batch-culture, and the preferred system was to add medium to a culture vessel regularly in small doses via a time-switch-controlled solenoid closure at a rate similar to the growth rate; a turbidimetric safety device ensured that cell density did not drop below levels permitting growth. A mixture of galactose or fructose (6 g./l.) and glucose (2 g./l.) allowed better pH control than glucose alone (6 g./l.). Doubling times of 14–16 hr. were obtained on occasion; the gas-phase oxygen concentration for fastest growth depended on cell density and was frequently less than atmospheric, suggesting that these cells can behave as microaerophils.

Microbiologists depend upon an adequate supply of a growth medium for their microbes, and intact animal cells seem likely to remain essential for *in vitro* growth of animal viruses for some time. Unfortunately, cell supply often limits the work accomplished, so that a simple and cheap laboratory-scale method for bulk growth of continuous cell lines has some attractions. A similar case can be made for cells required for virus vaccine manufacture.

This paper describes an experimental approach to such a method, based on a continuous flow of medium to a culture of suspended cells. Emphasis has been placed on economy of operation, both in materials and attention, and the method should be capable of expansion to pilot-plant scale or contraction to the needs of a small laboratory; its feasibility has previously been reported briefly (Cooper, Burt & Wilson, 1958). Some aspects of the growth conditions of the cells are reported, particularly their very rapid growth under optimal conditions with a doubling time of less than 16 hr., and the inhibitory effect of oxygen excess which could occur at oxygen concentrations less than that in normal air. The use of these cells for poliovirus bulk growth and assay will be described separately.

METHODS

General scheme. The object was to overcome the bottle-neck of cell production in stationary cultures as cheaply and simply as possible, and continuous suspension culture seemed the best method. However, some ancillary bottle culture was still needed, and a simplified procedure for this is described. Four methods for suspension culture were tried: batch culture, continuous medium flow ('chemostat'), photo-electrically controlled discontinuous medium flow ('turbidostat'), and a combination of the last two ('chemo-turbidostat'). Their merits are compared below. The apparatus used for the turbidostat and the chemostat were similar, and the differences involved are described where appropriate. As emphasized below, reliability was essential; all the components described have proved reliable in almost continuous operation at 37° for between 9 and 25 months.

The apparatus is shown in Fig. 1; where possible, standard commercial products were used throughout. Pyrex aspirators (20 l.) acted as medium reservoirs (*a*), culture vessel (*b*) and receiver (*c*); *b* and *c* were stirred and gassed continuously. Aspirators *b* and *c* were silicone-treated before autoclaving; this lessened but did not prevent the accumulation of some cells at the culture meniscus. Medium was allowed to fall at intervals via a solenoid-operated spring closure (*d*) (Callow & Pirt, 1956) controlled by a time-switch ('chemostat') or by a photo-electric device ('turbidostat'). These are described in Figs. 2 and 3. Alternatively, medium was continuously pumped (chemostat) at a rate monitored by a rotameter (*e*) from *a* to *b*, which overflowed via the constant-level vent into *c*.

The inset within the dotted line shows alternative flow control to the solenoid and includes the pump (*f*), which consisted of a rotating ring of rollers pressing the rubber tubing against a pivoted plate kept at a constant pressure by a spring. The pumping rate was governed by the speed of rotation, dimensions of the tubing, spacing between the rollers, tension of the spring and the head of medium, the latter necessitating a Marriotte tube in the reservoir to keep the hydrostatic pressure constant. Wear by the rollers on the tubing was considerable. The Marriotte tubes were not necesary for the turbidostat. A rotameter calibrated in 5–150 ml./min. for air was adequate for 0·2–5 l. medium/24 hr. Cells grew in *b* in a shallow layer of medium of constant depth (5–7 cm.), but the depth in *c* increased as cells plus medium entered. The overflow from *b* must be removed rapidly, and therefore intermittently, rather than by a continuous siphon, as otherwise cells settled in the pipe. Harvesting (P) and replenishing ports (P′) were dust-caps made of 37 × 55 mm. or 60 × 80 mm. aluminium screw-cap containers (Metal Box Co., London, W. 1) with stainless steel tubes bolted through, and could be thoroughly flamed when open or closed. Where possible all tubing and T-pieces, particularly those passing through bungs, were of stainless steel because of occasional accidental breakage of glass tubes. Outflows from culture vessel and receiver were $\frac{3}{8}$ in. internal diam., other tubing was $\frac{1}{4}$ in. internal diam. All pipelines in contact with medium were of silicone rubber tubing (Esco Rubber Co., Ltd., London, N. 16), and air-lines were of 'drab' white rubber tubing (J. G. Ingram and Co., Ltd., London, E. 9); closure was by screw clips (*g*) on to tubing protected by cotton-backed adhesive tape. Sampling (X) and inoculating (Y) ports employed $\frac{1}{2}$ oz. bottles (United Glass Bottles Ltd., Leicester Street, London, W.C. 2); the central threaded holder was a Hemmings filter (Hemmings Ltd., Beaumaris, Anglesey) with the centre perforated plate drilled out, and the top portion was an inverted $\frac{1}{2}$ oz. bottle with the bottom removed and a bung inserted. The magnetic stirrer motors (Baird and Tatlock Ltd., Chadwell Heath, Essex) had the cover-plates discarded and speed-adjusting rheostats removed for remote operation to avoid local heating; the stirrers were barrel-shaped, weakly

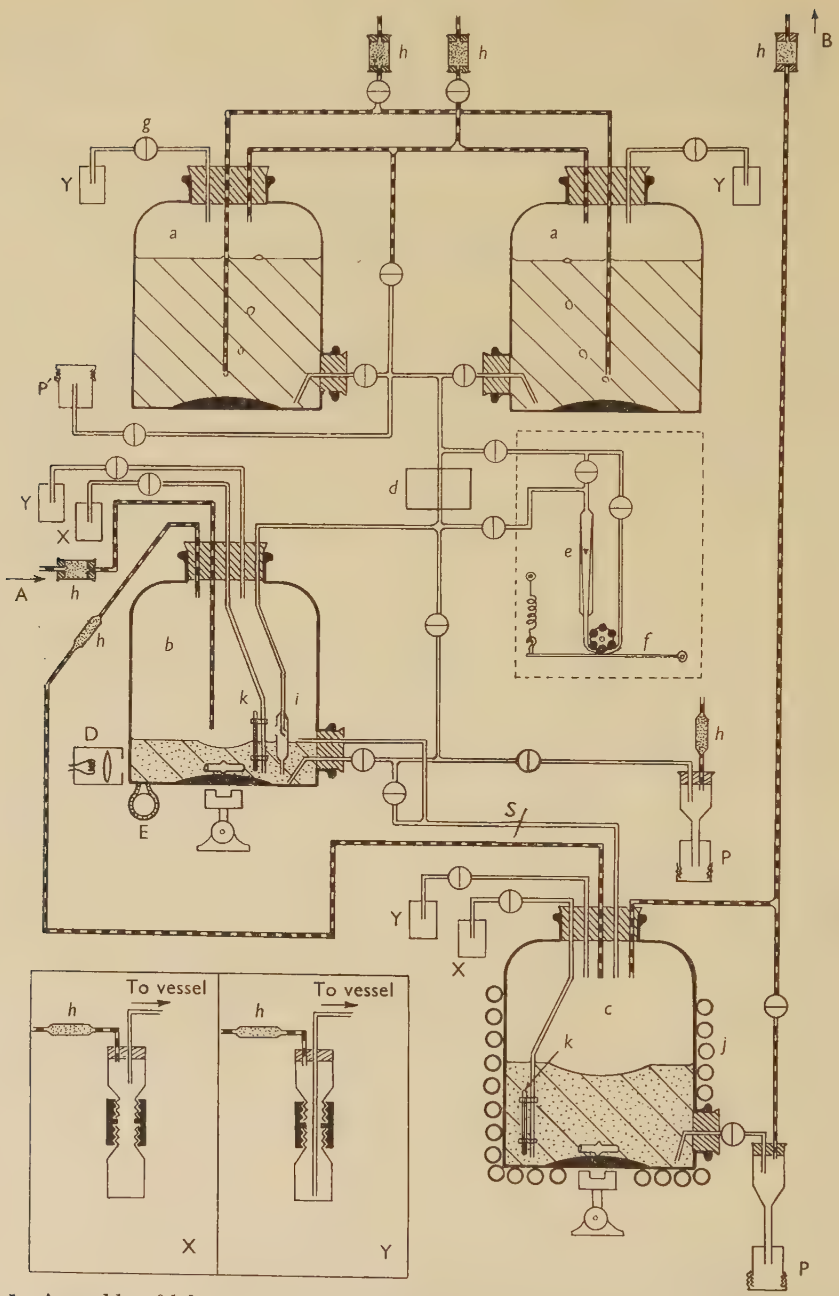

Fig. 1. Assembly of laboratory apparatus used for continuous culture of ERK cells. The lines carrying air or a gas mixture are stippled; *a*, medium reservoirs; *b*, culture vessel; *c*, receiver; *d*, solenoid valve closure; *e*, rotameter; *f*, pump; *g*, screw clips; *h*, cotton wool plugs; *i*, anti-splash medium inlet; *j*, cooling jacket of rubber tubing; *k*, thermometers; A, gas inlet; B, gas outlet; D, light source; E, photocell; P, harvesting and P′ replenishing ports. X and Y are sampling and inoculating ports respectively, shown schematically in the main diagram and in detail in the inset; samples were withdrawn via X or added via Y by sucking or blowing through the cotton wool filters. The bar across the medium line at S indicates the position of the sterile connection made during assembly.

magnetic stainless steel (Esco Rubber Co., London, N. 16) or strongly magnetic iron covered with polytetrafluorethylene (X-lon Products Ltd., London, S.W. 1). The latter were preferred, but they spin on a central tyre which wore flat in 3 weeks of continuous running; therefore the tyres were machined off and replaced with stainless steel rings. Apart from the sampling, harvesting, replenishing and inoculating ports, all openings to the air were protected by 2 × 15 cm. cotton-wool plugged tubes (*h*). To avoid surface bubbles which affected the photoelectric control, the medium inlet had an anti-splash device (*i*); it was preferred to keep the inlet visible to detect possible leaks through the solenoid closure. The gas outlet (*B*) was taken to the highest level possible to avoid spillage in case of undue leakage through the solenoid closure. The culture vessel (*b*) was covered with light-proof black cloth with lift-up flaps for inspecting stirring, volume and temperature. Thermometers (*k*) enclosed in *b* and *c* were tied to the sampling tubes with stainless steel wire so as to be away from the stirrer and more easily visible. The whole device was mounted on a frame in a room at 35°–36° with efficient air circulation; care was necessary to keep heat-producing electrical components away from the culture vessel, in which the temperature was 36°–37°. The receiver was cooled to between 28° and 32° by water pumped through a jacket of rubber tubing (*j*) from a water bath (Grant Instruments Ltd., Cambridge), which was cooled by copper coils through which mains water passed and was heated thermostatically to about 27°. A small inverted aspirator filled with water acted as a constant-level device for the water bath, to replace evaporation losses.

Stirring. Continuous stirring was essential; relatively slow rates were adequate to suspend the cells, but to promote gas exchange a shallow layer with as rapid stirring as possible without frothing was preferred. The culture vessel stirring rate was kept constant at 250–300 rev./min., using a 6 cm. stirrer, but the receiver stirring rate was increased as the depth of fluid increased. Extensive cell trauma was not found, and grinding was minimized by using a barrel-shaped or centre-tyred stirrer on the convex aspirator bottom; culture volumes of 1–2 l. rather than 100–200 ml. also minimized grinding, loss of cells on meniscus and difficulties of obtaining very low pumping rates. Even with such a shallow layer there was evidence that CO_2 removal was not adequate; this could probably be met by higher gas flow rates.

Aeration. A mixture of O_2, N_2 and CO_2 was obtained from cylinders with 'BOR 12' (British Oxygen Gases, Greenwich) regulators, which were sufficiently sensitive to control flow rate using a mercury manometer with fine capillary by-pass. The mixture, at a total flow of 100 ml./min. and monitored by a rotameter as a check on the manometers, was fed without deliberate moistening via a cotton-wool plug (Fig. 1, A) to 3 cm. above the culture surface; the oxygen demand of the culture would probably be met by 10 ml./min. Bubbling gas through the culture in the presence of antifoam agents was avoided because of risk to cell viability; varying the gas mixture could probably meet most oxygen needs.

Medium flow control. Flow in the chemostat was controlled by varying the speed of the pump or altering the duration or frequency of energizing the solenoid by the time switch; the difficulties in judging the culture's needs are discussed below. The flow in the turbidostat depended on changes in the light reflected by the culture which increased with increase in cells; an increase in reflected light decreased the resistance of the photocell beneath the culture vessel, and the relay operating the solenoid was actuated when the resistance fell below a pre-set value. The optical arrangement is shown in Figs. 1 and 2; two ORP. 90 cadmium sulphide photocells (Mullards Ltd., London, W.C. 1) were used to minimize effects of variation in light source intensity, resulting particularly from mains voltage variation, which had an appreciable effect on the light even with a constant-voltage transformer (Advance Components Ltd., Ilford, Essex). The light source was an intense light microscope lamp (C. Baker and Co., Croydon, Surrey) with a 6 V. 50 W. bulb with five filaments,

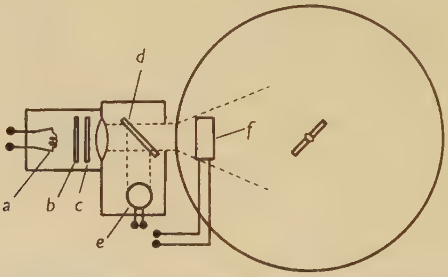

Fig. 2. **Plan of optical arrangement for turbidimetric control; the large circle represents the culture vessel and the central bar the stirrer. Light from the bulb (*a*) passes through heat-resistant (*b*) and red (*c*) filters and a focusing lens. Some light is reflected by a sheet of plain glass (*d*) on to the balancing photocell (*e*), and the remainder passes into the culture over the controlling photocell (*f*).**

so that failure of one filament would not inactivate the control, and was run at 75 % of rated voltage to increase filament life. As shown in Fig. 2, the light beam entered a light-proof box through a heat-resisting (*b*) and a red (*c*) filter (no. OR/2, Pilkington Glass Co., London, S.W. 1) and was partly reflected by a plain glass plate (*d*) on to the balancing photocell (*e*); most of the light entered the culture from the side and was reflected by the tissue cells on to the photocell beneath (*f*). Two alternative electrical control systems were used; one (Fig. 3*a*) embodied a Multilec controller-recorder (George Kent Ltd., Luton, Beds.) with a knob which could control cell density, coupled to the output of a stable pH meter (Model 23 A, Electronic Instruments Ltd., Richmond, Surrey); the other (Fig. 3*b*) used a transistor relay (no. 596 HS (C)/TS. 5205, Magnetic Devices Ltd., Newmarket, Suffolk) across a Wheatstone's bridge, the cell density being varied by the variable resistances. Either system operated the solenoid closure on the medium line via a Sunvic (Sunbury, Middx.) relay no. F. 102/3 M. The first was used to establish the conditions for control from a continuous record, the second was a cheaper alternative suitable for duplication. A third and simpler but less stable circuit (Fig. 3*c*) was used to control turbidity in the 'chemo-turbidostat' using additionally a time switch (type MZM, Venner Ltd., New Malden, Surrey). An intermittent solenoid closure was preferred to a peristaltic pump, as there was much less wear on the tubing and it was easier to buy or build. A forced flow (e.g. by a pump) rather than continuous slow gravity flow was essential, as with such very slow flow rates gas bubbles constantly blocked the medium lines; no such trouble occurred with the rapid intermittent flows from the solenoid valve.

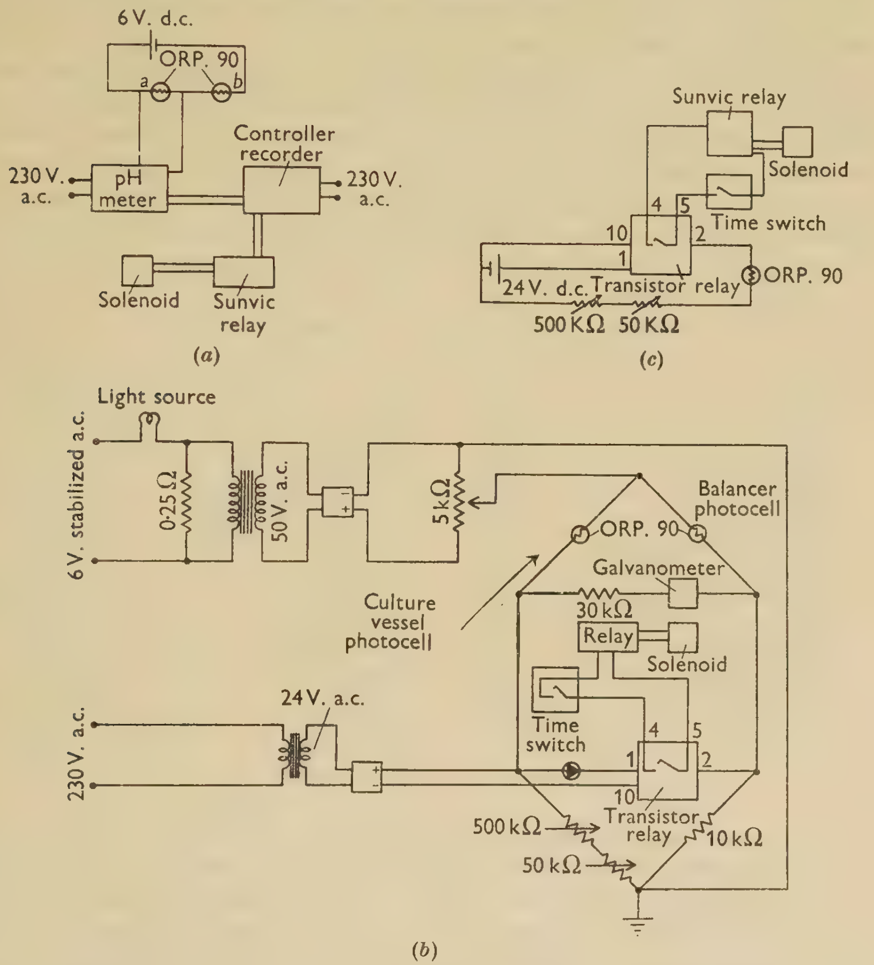
6 V. d.c.
ORP. 90
a
b
230 V. a.c.
pH meter
Controller recorder
230 V. a.c.
Solenoid
Sunvic relay
(a)
Sunvic relay
Solenoid
4
5
Time switch
10
2
1
24 V. d.c.
Transistor relay
ORP. 90
500 KΩ
50 KΩ
(c)
Light source
6 V. stabilized a.c.
0·25 Ω
50 V. a.c.
5 kΩ
Balancer photocell
ORP. 90
Galvanometer
Culture vessel photocell
30 kΩ
Relay
Solenoid
24 V. a.c.
230 V. a.c.
Time switch
4
5
1
2
10
500 kΩ
Transistor relay
10 kΩ
50 kΩ
(b)

Fig. 3. Electrical circuits used for the turbidimetric devices. (*a*) Shows the controller-recorder actuated by the pH meter; of the twin ORP. 90 photocells *a* is the balancer and *b* is beneath the culture vessel (see Fig. 2). (*b*) Is the balanced circuit suitable for independently controlling the turbidostat in which the photocells are arranged as in (*a*). (*c*) is the simplified circuit used as a cut-out device for the chemo-turbidostat; the numbers show the connexions for the transistor relay, and the photocell ORP. 90 is placed beneath the culture vessel as in Fig. 1.

Medium. Advantage was taken of the considerable literature on media for cell culture (e.g. Morgan, 1958). However, most of such work has justifiably aimed at simplification, tending to 'minimal' media, but we have aimed at a 'maximal' medium (medium CSV.6, Table 1) giving fastest growth and highest cell densities as cheaply as possible. Earle's saline was used as a basis (Earle *et al.* 1943) in which the salt concentrations are very similar to those found optimal by Eagle (1956). Lactalbumen hydrolysate (0·5 mg./ml.) was roughly equivalent to a 10- to 20-fold Eagle's (1955) amino acid supplement if extra glutamine or glutamic acid plus NH_4Cl and methionine were added; extra arginine (Thomas, Ziegler, Schepartz & McLimans, 1958) and inositol were also included together with extra glucose and bicarbonate. The freezing-point of the medium was $-0{\cdot}55°$. Most components were added to their highest non-toxic levels; the contents of K^+ and PO_4''' should theoretically limit yield to about 2×10^7 cells/ml., but this level has not been reached (mainly due to difficulties with pH control and glucose supply); and so no simplification has yet been attempted. Unlike the experience of Ziegler, Davis, Thomas & McLimans (1958), bicarbonate could not be omitted because of the high acid production by our cells; the other substances with appreciable buffering activity, lactalbumen hydrolysate and phosphate, were at their maximum non-toxic concentration. Although high cell density and ease of operation were the major aims, the growth rates achieved when gas mixtures were optimal were sometimes very rapid. All water was distilled in one step from hard mains water by a glass still (Loughborough Glass Co., Loughborough, Leics.). Horse serum, obtained from selected horses by venepuncture, and calf serum from a local abattoir, were Seitz-filtered without heat-inactivation before being added to the medium, which was then filtered through EK or EKS grade pads (Carlson Ltd., London). With this treatment no pleuro-pneumonia-like organisms were found in our cultures. Since medium often stood at 37° for several weeks, the penicillin level is high in CSV. 6; nystatin (Mycostatin, Squibb and Co., Ltd., Speke, Liverpool) was occasionally added sterilely to 25 u./ml. after filtration but was not added routinely to the reservoirs because of possible accumulation of toxic oxidation products (Paul, 1959); otherwise all mixing was done before filtration.

Table 1. *The components of medium CSV.6*

	g./l.		g./l.
NaCl	5·48	Biotin	0·001
KCl	0·5	Choline chloride	0·001
$CaCl_2$	0·2	Folic acid	0·001
$MgCl_2.6H_2O$	0·2	Nicotinamide	0·001
NH_4Cl	0·05	Calcium pantothenate	0·001
$NaHCO_3$	2·5	Pyridoxal 5′ phosphate	0·001
$NaH_2PO_4.2H_2O$	0·5	Thiamine	0·001
		Riboflavine	0·0001
Difco yeast extract	1·0		
Lactalbumen hydrolysate	5·0	Penicillin	375,000 units/l.
Glucose	6·0	Streptomycin	0·125
L-Glutamine	0·1	Neomycin	0·10
L-Glutamic acid	0·3	Phenol red	0·01
DL-Methionine	0·1	Water	To 1000 ml.
L-Arginine hydrochloride	0·25	Horse serum	75 ml.
Inositol	0·01	Calf serum	25 ml.

The sera were added after making up to 1 l.

Ancillary bottle culture. This has been retained to restart a culture machine with minimum delay in event of breakdown; otherwise cells were stored at -70° in 20 % (v/v) glycerol in horse serum, or as monolayer cultures at 31° with weekly medium changes, in which case subculture was necessary every 6–8 weeks. Screw-capped bottles (20 oz. medical flats, United Glass Bottles Ltd.), with a culture area of 75×160 mm., were preferred to 1 l. Pyrex Roux flasks with bungs for their ease of handling and cheapness; the glass is slightly 'alkaline' but is flatter than the available Pyrex. On average the bottles were used ten times and then rejected. Medium CSV.6 was also satisfactory for stationary bottle culture, giving yields of up to 10^6 cells/ml., and a tenfold cell increase per week without medium change if inoculum and initial pH were correct (see below); use of 0·11 % bicarbonate, 0·1 % glucose and a less alkaline starting pH gave a faster growth rate (20-fold per week) but required a mid-week medium change. Routinely 50 ml. of medium were added per bottle (initial pH 6·7, equilibrating without gassing to pH 7·4) followed by $4 \pm 0{\cdot}2 \times 10^6$ cells, and the bottles were left at 37° for 7 days before harvesting with 2·5 mg. trypsin/ml. (Difco) in phosphate-buffered saline (Dulbecco & Vogt, 1954). Two separate lines were maintained, and some bottles were placed at 31° without subculture in case of loss by contamination. Yield was $2\text{–}5 \times 10^7$ cells/bottle. Bottles when empty were autoclaved in 2 % (v/v) saturated solutions of sodium hexametaphosphate and sodium metasilicate in tap water, mechanically brushed with brushes made specially for these bottles (Matburn and Co. Ltd., London, W.C. 1), rinsed inside and out with cold tap water, N-HCl, tap water again and finally four changes of distilled water before drip-drying. Care was taken to prevent drying before the final stage. It is believed that this is one of the simplest effective procedures; the alkaline treatment in time caused a 'bloom' on the glass which hindered cell growth and caused bottles to be rejected. Individual 'toxic' or 'greasy' bottles were rejected forthwith; routine rejection of a cheap bottle was preferred to varying the washing procedure.

Operation of culture machine. The entire apparatus was autoclaved containing a few ml. of water for $1\frac{1}{2}$ hr. at 10 lb. in.$^{-2}$ in two parts, then mounted on its stand and the sterile connexion made (Fig. 1, S). Medium (20–40 l.) was pumped through a sterile filter-press via a sterile connexion (comprising an aluminium dust cap, Fig. 1, P′, fitted inside with a short length of silicone tubing) into the reservoirs, and enough medium was run into the culture vessel to overflow. Four to six 20×20 cm. Carlson filter pads (EKS grade) were used and were pre-washed with 8 l. distilled water and 1 l. of medium; finer grades than this sometimes gave media yielding low growth rates. When filtration was finished, in order to avoid a liquid bridge the medium in the inlet line was drained out before disconnecting the filter press by opening the screw clip leading to the reservoir air-space. The stirring speed was increased almost to frothing, and the gas mixture started at 10 % CO_2 and 15 % O_2 and left overnight to equilibrate. The cells trypsinized from 20–40 bottle cultures were added next day to give $4\text{–}6 \times 10^5$ cells/ml. in the culture vessel, and were followed by mycostatin to give 25 u./ml. The pH was controlled by varying the CO_2 content of the gas phase. Cell concentrations were determined by haemocytometer count on four replicate samples of 30–200 cells each in the presence of 2 mg. trypan blue/ml., blue-staining cells being classed as non-viable. With the chemostat, the cell count and the pH (as described below) were followed on a daily sample, and the gas and medium flow rates were adjusted daily to maintain optimum conditions; gas bubbles had to be dislodged from the pipelines. The turbidostat and chemoturbidostat, once set at optimal growth rate and cell density, needed a daily check on volume through-put and operation of components and a less frequent cell and pH check; cells in the receiver were fed twice-weekly with 1–2 l. medium. The pH was affected by both CO_2 and O_2 levels in the gas phase as well as by growth rate and cell density. Cells were harvested once-weekly from the receiver via the lowest port, and in order to avoid a liquid bridge the medium in the lower part of the port was drained out by opening the screw clip leading to the gas outlet. Occasional difficulty was found with the gradual formation of very large clumps which did not overflow from the culture vessel; these were siphoned off when necessary.

Cell strain. A transformed line of embryo rabbit kidney (ERK) cells was used. This was isolated by Westwood, Macpherson & Titmuss (1957) and grew well in medium CSV.6 after one or two subcultures in this medium in our laboratory.

Cell state. The microscopical appearance of the cells was a useful indication of culture conditions. Oxygen lack or low pH led to a granular cytoplasm and irregular outlines with a tendency to form syncytia; slight oxygen excess gave large clear well-defined cells, becoming very large in dangerous oxygen excess. Trypan blue staining was a good qualitative indication of cell viability (quantitative correlations were not made), but cells generally became trypan blue-positive only when it was too late to remedy the damage. A disadvantage was that the cells often grew in clumps; on a few occasions these became very large (*c.* 1 mm. diameter).

RESULTS

Batch growth of ERK cells in stirred suspensions

Many reports now describe growth of animal cells in suspension, and this was readily confirmed by us (Fig. 4) using a simple stirred pot gassed with 5% (v/v) CO_2 in air. These experiments showed that the cells grew rapidly on occasion but growth rate fell markedly above *c.* 2×10^6 cells/ml., and the maximum density obtained was 5×10^6 cells/ml. This limiting density depended on the surface area for a given volume of medium. Addition of glucose and arginine plus inositol (McLimans, personal communication) or a medium change sometimes but not always restored growth. Cell growth rarely started well below 2×10^5 cells/ml. It was therefore clear that the optimum growth range for animal cells was narrow and was rapidly exceeded in culture, and unless good conditions were rapidly restored the cells entered a lag which was usually long or irreversible. Cell viability was very variable. Such growth also required daily attention if good yields were desired, and it was felt that a continuous culture, run under steady optimum conditions, would be more satisfactory. The following section describes attempts to obtain this steady state by adding medium at a constant rate ('chemostat').

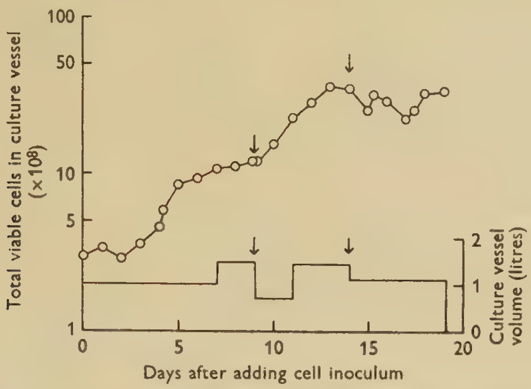

Fig. 4. Rate of growth of ERK cells in batch suspension culture. Changes in volume indicate supplements of fresh medium or complete medium changes (arrows).

Growth of ERK cells in suspension with continuous medium flow ('chemostat' principle)

Fig. 5*a* shows the results of the longest of several runs in which growth was continuously maintained by uninterrupted medium flow. Herbert, Elsworth & Telling (1956) have shown that this type of system with bacteria is much more efficient than batch culture; this seems to apply also to animal cells. Over a period of 4 months, a culture-vessel volume of 1·5 l. yielded an average of 10^9 cells per day in 1 l. of medium; this was a concentration rarely reached in bottle cultures with the same medium and a rate of yield not obtained in batch suspension culture. The maximum cell density was 2×10^6 cells/ml.; cell viability was 90–95 %. The growth rate was also high, as the doubling time averaged 25 hr. over the whole period and was occasionally less than 16 hr. Figure 6 shows that when cells were removed from the culture vessel and chilled, growth recommenced almost synchronously with a very short doubling time (14 hr.) if conditions were optimal. Most suspension cultures probably started synchronously, presumably because the cells cool during harvesting.

Unfortunately, although continuous medium flow was more efficient than batch growth, it was more troublesome to operate because the system was not self-balancing and not, therefore, a true chemostat although set up on the chemostat principle. As with bacteria, the cells were washed out of the culture vessel if a constant flow rate exceeded the growth rate; if the growth rate exceeded the flow rate, however, the cell concentration increased to a limiting value (usually with a very low pH) and the cells then entered a lag (i.e. growth was not restored by immediately restoring conditions to optimal values) so that the flow rate became excessive and the cells were rapidly

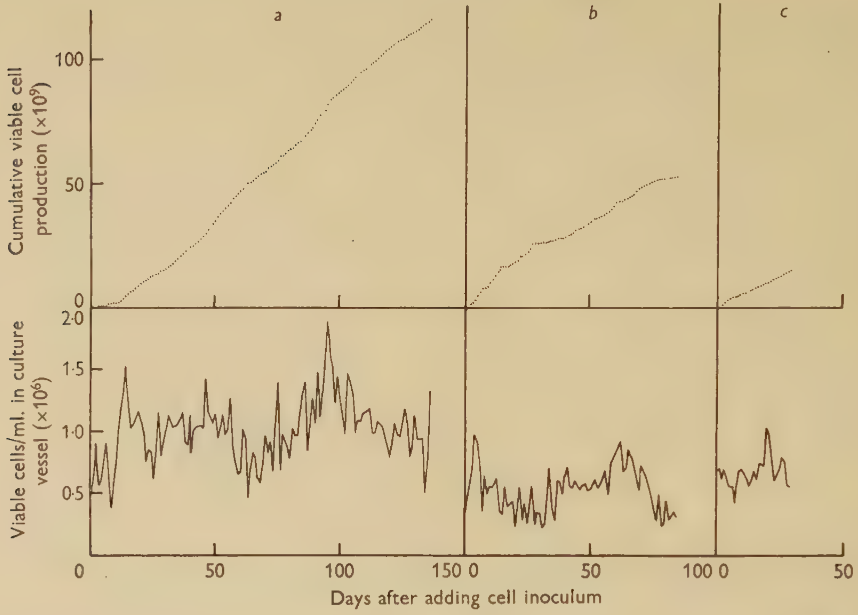

Fig. 5. A comparison of the yields and culture-vessel contents in continuous suspension culture of ERK cells during: *a*, a typical chemostat run; *b*, a typical turbidostat run; and *c*, a typical chemoturbidostat run.

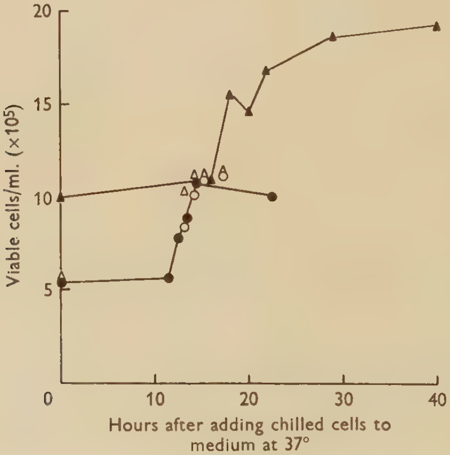

Fig. 6. Synchronous growth from cells adapted to grow in suspension. Cells from the culture vessel during the run shown in Fig. 5(*a*) were centrifuged, shaken gently at 0° for 1·5 hr. and diluted in 100 ml. medium CSV.6 at 37° in a stirred and gassed culture vessel. Gas mixtures (v/v) were 9 % O_2, 5 % CO_2 and 86 % N_2 (Expts. ○, △, ●) or 12 % O_2, 5 % CO_2 and 83 % N_2 (▲).

washed right out. As mentioned below, oxygen tension in the liquid phase (governed by oxygen tension in the gas phase and cell concentration, other factors being constant) also critically affected growth rate. It is possible that the culture would reach equilibrium if left to itself, but the narrow growth ranges, particularly of cell concentration and pH, make this unlikely; any attempts to allow equilibration were soon terminated as the culture would clearly soon be lost. Therefore, it was necessary every day without exception to anticipate the needs of the culture in the following 24 hr., and make adjustments accordingly. On two occasions a steady state was kept for some days, probably because oxygen tension happened to restrict growth rate to the same value as the flow rate.

We therefore conclude that with medium CSV.6 and ERK cells, which do not completely oxidize a large proportion of the available glucose, a culture machine running on the 'chemostat' principle is not a very satisfactory system. The main reason for this is probably that the cells can use glucose at a very high rate whether they grow or not, and glucose restriction does not much restrict growth without harming the cells. Other factors may contribute, however, particularly the difficulty of maintaining cell concentration, oxygen tension and pH in their narrow optimal ranges. Possibly a differently balanced medium or a rate-limiting component can be found which when deficient will slow growth without damaging the cells; meanwhile, a better self-regulating system was desired, and the approaches to this are described in the following section.

Growth of ERK cells in suspension with automatically controlled medium flow

Three methods of automatically controlling medium flow seemed feasible, based on (*a*) redox potential, (*b*) pH, (*c*) cell density.

Redox potential. Since growth rate depended on oxygen tension, growth should always be optimal if the oxygen tension or redox potential in the culture was poised at the optimal value. Two experiments were therefore conducted with an immersed platinum electrode and an isotonic saline-agar bridge leading to an external saturated KCl-calomel electrode. The e.m.f. of this cell measured with a pH meter was *c.* +200 mV. with medium only, but was unchanged by the addition of cells, whatever their state of growth. Presumably either the electrode polarized easily, or the high redox potential inside these cells (Cooper, 1959) does not encourage development of the very reducing environment encountered with bacteria (Hewitt, 1950). This method was therefore abandoned for control purposes.

pH. It proved feasible, using a demountable glass electrode (no. GSE. 23, Electronic Industries Ltd., Richmond, Surrey) to mount a glass electrode, with an internal platinum electrode but without the internal buffer solution, inside the culture vessel before autoclaving. After autoclaving the entire apparatus and allowing it to cool, the internal buffer solution was passed via a very fine silicone rubber tube, previously inserted, into the inside of the glass electrode. Using the same agar bridge as before, the system was calibrated for pH by measuring the e.m.f. of the glass-calomel pair when the medium in the culture vessel was without cells and in equilibrium with its gas phase (after 24 hr.). A sample of culture-vessel medium was then withdrawn, and while still at 37° gassed with the gas effluent from the culture vessel in a small non-sterile assembly which contained glass and calomel electrodes, themselves previously calibrated with standard buffer. The pH meter recording the pH in the culture vessel was then set to the true pH as determined on the withdrawn gassed sample.

This method showed that the pH of very recently withdrawn samples before gassing was about 0·1 pH unit higher than after gassing, and that visual estimates of pH from the phenol red indicator and with pH test-papers (British Drug Houses, Poole) gave agreement within 0·2 pH. The pH was routinely determined either with a pH meter or visually and with test papers on samples, but before opportunity for cooling and CO_2 loss had occurred. Cells briefly tolerated pH ranges of at least 6·6 to 7·4, but did not grow well unless within pH 6·9 to 7·1. This confirms the findings of Kuchler & Merchant (1956) with growth in suspension; estimation of the true pH near the cells is difficult in stationary bottle culture, where the pH in the bulk of the medium is about pH 7·3 for optimal growth. Use of the controller-recorder showed that a small alteration in the CO_2 content of the gassing mixture caused a detectable pH change in a few minutes, but the new pH value was not established fully for about 6 hr.; the half time was about 1 hr.

Once calibrated, the internal electrode accurately reflected changes in pH of the culture for several weeks at least. However, for simple measurement of pH this method had several disadvantages; (*a*) re-calibration was lengthy and was necessary 2 or 3 times a week; (*b*) initial assembly was difficult, particularly in obtaining satisfactory agar-bridges, since it was felt necessary to keep the saturated KCl from immediate contact with the culture; (*c*) breakage rate of glass electrodes was high; (*d*) while one electrode ran for 1600 hr. without trouble in the hands of Callow & Pirt (1956), others were less satisfactory. Since, as mentioned below, long runs are essential for our purpose (preferably > 1600 hr.) it is essential that any control system either be very reliable, or be external for replacement without stopping the culture. Its disadvantage as a control system was that maintaining the pH at 7 units was not by itself sufficient to promote rapid growth; this coupled with difficulty of operation led to our abandoning the immersed glass electrode both for pH estimation and growth control.

Cell density. Photo-electric control of the turbidity of a cell suspension (turbidostat) works well for bacteria (Bryson, 1952), and a similar system was accordingly applied to the apparatus described in Fig. 1. The principle is that an increase in cell density caused by growth increases the turbidity of the culture above a pre-set value and this automatically causes fresh medium to flow into the culture. The cells are thereby diluted until another pre-set turbidity value is reached, when the medium flow is automatically switched off again. Small mains voltage variations gave large variations in light intensity, and failure of the bulb or lighting circuit inactivated the control, or left the solenoid valve open so that medium flowed unrestrictedly; these difficulties were met or minimized by a double-photocell system, a multifilament bulb and the circuits shown in Fig. 3. Some cells settled on the glass over the photocell, but these were removed when necessary by scraping with the magnetic stirrer moved by a magnet held in the hand.

Two machines ran for 80 and 85 days respectively as turbidostats and the total cell production is shown for one (Fig. 5*b*). As may be expected, culture vessel contents were more stable than in the chemostat but the control was affected by non-viable cells, size of cells, and the turbidity which forms with stirring in absence of cells. Cell viability was 80–90 %, in general somewhat less than with the chemostat. When conditions were optimal the growth was quite rapid, but the cultures recovered from periods of suboptimal conditions with difficulty and the cell density tended to drift to low values.

This revealed one fundamental disadvantage of the turbidostat as a system for culturing micro-organisms; unlike the chemostat, it has no intrinsic 'incentive to growth', and relies entirely upon one addition of medium producing sufficient growth to stimulate a second addition. The cell concentrations desired were close to the maximum supported by the medium, and these cells can use much glucose and possibly other substrates without necessarily growing; if in lag or suboptimal conditions, or in high cell density, they may not grow sufficiently to stimulate the mechanism before their glucose supply is exhausted, and the culture will be lost unless detected in good time and given a supplement of glucose, or a medium change, or deliberately diluted. Thus a pure turbidostat could be used but was not entirely satisfactory; the following section describes a combination of both chemostat and turbidostat principles.

The chemo-turbidostat

The chemostats in general gave good growth, and their main disadvantage was that literally daily attention was necessary and an error in judging in advance the medium flow rate in relation to cell growth rate sometimes rapidly lowered the cell density below levels permitting growth, or increased it to values at which the cells entered a lag phase. Therefore, several chemostat runs were made in which this snag was met by using the turbidimetric control to stop the supply of medium if the cells were diluted below a certain pre-set minimum. For this, medium was continuously passed in small intermittent doses by means of a time switch actuating the solenoid closure, at a rate if anything slightly greater than the growth rate. The photocell arrangements of Figs. 1 and 2 and the circuits of Fig. 3*b* or *c* were used.

Such a system appeared fairly satisfactory, combining 'incentive to growth' with a reliable safety device, and requiring only a rough initial balancing of growth rate and medium input and much less routine attention. Fig. 5*c* shows a run with this system, additionally using a fructose- or galactose-glucose carbohydrate source. Its cell-production rate averaged the same as the turbidostat but was more stable.

Variation of carbohydrate source

A difficulty in the continuous running of cell-culture machines with the ERK cell was the large amount of non-volatile acid produced. Eventually the cultures often became too acid for growth even with no CO_2 in the gas phase, necessitating a medium change or addition of bicarbonate. It was hoped to avoid this by replacing glucose by galactose (Eagle, Barban, Levy & Schulze, 1958), but four attempts (two in stationary and two in continuous suspension culture) failed to subculture cells indefinitely in CSV. 6 containing galactose in place of glucose; the cultures died after 10–20 generations. However, fructose alone, or a mixture of galactose or fructose (6 g./l.) and glucose (2 g./l.) appeared to carry the cultures well with good growth and less production of non-volatile acid.

Receiver conditions

As it was convenient to harvest the cells only once-weekly, some storage system was needed. Efficient running would entail a depletion of the environment in the culture vessel, so that without attention the cells overflowing and collecting in the receiver may be in poor condition after 7 days. This was found to be so, and was remedied in part by maintaining the receiver at 30°, and supplementing with fresh medium as follows: after harvesting and draining out all cells, 1–2 l. of fresh medium were run into the receiver from the reservoir as an initial feed, followed by 1–2 l. on the third day and a further 1–2 l. on the sixth day, the cycle being completed by harvesting on the seventh. Viability was then about the same as in the culture vessel. This method, however, is not entirely satisfactory and there is evidence that the cells obtained are not in as good condition as rapidly growing ones; this aspect therefore requires more study. The cooling was insufficient to stop growth completely so that some additional cells were produced by growth in the receiver; sometimes overall yield was deliberately increased by large feeds and by increasing the receiver temperature, but this expedient was inefficient.

The effect of oxygen tension on growth rate

Fig. 5*a* shows that the culture vessel content during the chemostat run varied widely from day to day; this was because the growth rate also varied. As may be expected, one factor affecting growth rate was the gas phase oxygen tension; growth was slowed by both oxygen lack and excess, the latter sometimes occurring at concentrations less than atmospheric. On two occasions during nearly steady states (Fig. 7) a small increase in oxygen concentration (from 22 to 24 %, v/v, at day 37) halved the growth rate, and a small decrease (from 19 to 17 % at day 45) increased the growth rate by 50 %. In other experiments higher concentrations (25–30 %) were rapidly cytocidal.

Presumably the important factor is the O_2 concentration in the liquid rather than the gas phase, and this will depend on cell concentration among other factors. Figure 7 gives the relations between growth rate, oxygen concentration and cell concentration during two portions of the chemostat run shown in Fig. 5*a*, and shows that growth rates frequently passed through maxima in a cyclic manner. A plot of cell concentration against oxygen tension for the 24 hr. periods during this run when growth rates were maximal (Fig. 8) shows that the two are related, and the extrapolation to zero cell concentration suggests that the optimal oxygen concentration in the liquid phase is equivalent to equilibrium with about 9 % (v/v) oxygen in the gas phase. This concentration is probably not absolute, however, and may, for example, depend on the catalase content of the serum (Lieberman & Ove, 1958), but the curve was useful in selecting oxygen tensions for optimal growth at given cell densities. Since the cell concentration varied, the liquid-phase oxygen tension should also vary, and this variation presumably caused the changes in growth rate since other conditions were steady; as growth rate in turn affected cell concentration, it is not surprising that conditions were cyclic.

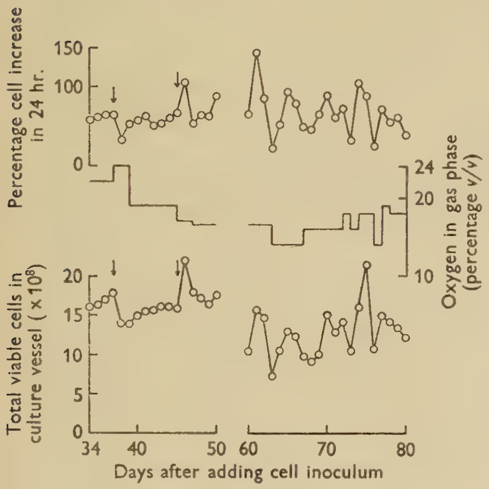

Fig. 7

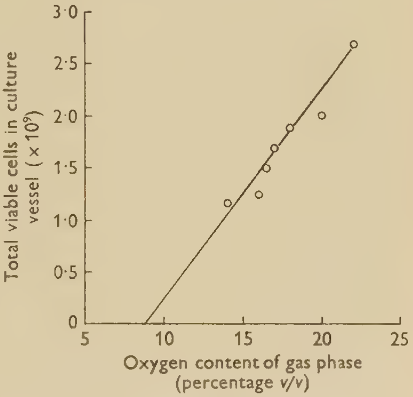

Fig. 8

Fig. 7. Abstracts from the chemostat run of Fig. 5*a*, showing effects of abrupt oxygen gas-phase changes during steady state (arrows) and cyclic response of growth rate and culture-vessel content.

Fig. 8. Relation between gas-phase oxygen tension and average culture vessel cell content for all periods when the daily percentage cell increase exceeded 87 % (probable mean doubling times of 24 hr. or less) during the chemostat run shown in Fig. 5*a*.

Osgood & Krippaehne (1955) reported that cells on an inclined immersed microscope slide grow best 1–2 cm. below the surface. Apart from this, the gas phase of tissue cultures has been relatively neglected, but several authors have recently reported that oxygen can be in inhibitory excess at quite low concentrations (Cooper, Burt & Wilson, 1958; Zwartouw & Westwood, 1958; Lieberman & Ove, 1958). Oxygen tensions are also low in tissues *in vivo* (Davies & Bronk, 1957). Lieberman & Ove (1958) state that the oxygen toxicity, particularly marked with single cells, can be overcome with catalase. The inhibitory effect of oxygen may be complex, however, and may perhaps only apply if cells are partly anaerobically adapted; for example, cells in roller tube culture are probably highly aerobic most of the time, yet grow well.

DISCUSSION

The term 'continuous culture' is often applied to growth of micro-organisms with continuous medium flow, but is also sometimes applied to batch growth of animal cells when unlimited subculture is achieved. We assume unlimited subculture and use 'continuous culture' for continuous medium flow with suspended cells (chemostat or turbidostat), 'batch-culture' for discontinuous culture of suspended cells and 'bottle-culture' for the necessarily batch growth of monolayers on glass. The terms 'deep' and 'submerged-fermentation' are used in the fermentation industry to distinguish suspended culture from culture on or near the liquid surface; although occasionally used for suspension cultures of animal cells, these terms are avoided here as the distinction does not apply to our system. It should be noted also that for convenience we use the term 'chemostat' for an apparatus set up on the chemostat principle, although in practice it rarely functioned as such.

Several different types of assembly have been used for successful batch-culture of suspended animal cells (Graff & McCarty, 1957; references in Ziegler *et al.* 1958). In starting the present work we accepted the principle that the simplest system is the stationary 'stirred-pot'. This is general in the fermentation industry; particular advantages are its ease of construction and scaling up or down.

A second principle is that continuous culture can be more efficient and less troublesome than batch-culture (Herbert *et al.* 1956). We modelled our system where possible on those satisfactory for bacteria (Herbert *et al.* 1956; Elsworth, Meakin, Pirt & Capell, 1956), while using ordinary laboratory equipment and allowing for differences in the cells used, animal cells being (*a*) more easily sedimented, (*b*) physically more fragile, (*c*) more sensitive to adverse chemical environment and (*d*) much slower growing than bacteria. For the last reason, and also because of the complexity of the medium, no theoretical studies were attempted and we assumed that volume change and growth rates at steady state are related as for bacteria (Herbert *et al.* 1956). As an example of the difficulties of theoretical work, Herbert *et al.* allowed their bacterial cultures 24 hr. to equilibrate after changing conditions slightly; an equivalent time for animal cells would be more than one month.

A third principle is that the apparatus must be reliable in every sense. Where possible, the cheapest and simplest laboratory apparatus has been used, except where apparent simplicity may affect its continuous running. This is because small mechanical failures were often lethal to the cells, and chance contamination has not been countered by any means except dismantling and re-sterilizing, except in two cases where small moulds found growing inside the reservoirs were completely inactivated by adding mycostatin (500,000 u./20 l.). Since dismantling, cleaning and re-sterilizing took several days, growth of a fresh inoculum (10^9 cells) sometimes took a week, and the cultures once started took 1–2 weeks to reach high yields again, the system was uneconomical unless at least 2 months continuous operation could be expected. However, sources of unreliability were eliminated once found and the components described have been quite reliable; the main chance of breakdown lay in contamination during inoculation or replenishing medium in the reservoirs. Harvesting and sampling were adequate with the devices described. Much protection was probably afforded by the antibiotics; however, because of its higher growth rate, once an antibiotic-resistant variant appears the contaminant will be much more likely to take over the culture than in bacterial chemostats (Powell, 1958).

Thus with continuous culture of animal cells, if one needs a large yield one also needs a relatively complex assembly, but a smaller-yielding apparatus could be much more simple. It is likely that the cell needs of a small diagnostic or research laboratory could be met by a small apparatus with a culture-volume of 300 ml., a simplified chemo-turbidostat (Fig. 3*c*) and a slow gas supply (10 ml./min.) from a 5% (v/v) CO_2-air cylinder. This should run for several months with little attention, although it seems unlikely that the yield-rate would be optimal. On the other hand, the large cell needs of a commercial plant producing vaccines might well be met by one 10 l. capacity fermentor of a design commercially available. The virus production by these cells will be considered elsewhere, but a single fermentor should be able to match the poliovirus output of most vaccine organizations if the objection regarding possible malignancy of cells were overcome; this objection may not apply to agricultural animal virus vaccines. A more economical medium than CSV. 6 could almost certainly be found.

In practice, when differences in cell concentrations were allowed for, the yield rates of the three types of assembly described differed by less than two-fold, although the actual yield of the chemostat arrangement was greater than the yields of the automatically controlled devices, partly because the attention the former demanded ensured that conditions were more often better and because the latter tended to drift towards lower cell concentrations. Despite this, however, an automatically controlled chemo-turbidostat is much to be preferred for its ease of operation; present work is directed to increasing practical culture-vessel cell populations.

We are indebted to the National Fund for Poliomyelitis Research, by whose generosity the Virus Culture Laboratory was established and this work supported. We are also grateful to Dr F. Sheffield for the original gift of the ERK cells, and to Dr E. Klieneberger-Nobel for testing our cultures at intervals for pleuropneumonia-like organisms.

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The Stimulatory Effect of Light on Nucleic Acid Synthesis in the Mould *Blastocladiella emersonii*

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SUMMARY: The fungus *Blastocladiella emersonii* possesses neither chlorophyll nor coloured carotenoids; yet, visible light induces increased nuclear multiplication, increased synthesis of DNA relative to total nucleic acid, and detectable changes in the soluble phosphorus pool of the fungus. The stimulatory effect is detectable through ontogeny, from the first few hours after germination to the end of the generation time of *c.* 15 to 25 hr. (depending on temperature). Light could be replaced by providing the plants with thymidine or thymine, but not with the corresponding RNA base, uracil. The DNA of *B. emersonii* contained the four principal bases usually associated with this nucleic acid. Finally, the interrelationships between DNA and RNA, and the effects thereon of nucleic acid analogues and inhibitors, were also studied. In particular, it was found that the DNA per cell doubled just before the first mitosis, while the total nucleic acid concentration did not change. The results of the latter experiments were interpreted in terms of the transformations which occur in the RNA-rich nuclear cap in the spores of the fungus during germination.

Upon germination, the spores of the water mould *Blastocladiella emersonii* may develop along three, distinctly different, ontogenetic paths (Cantino, 1959; Cantino & Turian, 1959): one leads to the formation of brown, thick-walled, resistant sporangial plants (R.S. plants); a second leads to ordinary, thin-walled, colourless plants (O.C. plants); and a third leads to thin-walled, orange plants (O. plants). It has been established that visible light accelerates growth along all three developmental vectors (Cantino, 1957). The purpose of this paper is to provide further data about the stimulatory effect of light upon the thin-walled, colourless plants.

The O.C. plants of *Blastocladiella emersonii* possess no detectable amount of pigmented carotenoids or chlorophylls; in spite of this, they grow more rapidly in the light than in the dark, and they fix more CO_2 per unit weight of organism under these conditions (Cantino & Horenstein, 1956). Thus, from the point of view of both comparative biology and comparative biochemistry, *B. emersonii* seems to occupy a unique position in the general scheme of living things.

Our previous studies, aimed at elucidating the mechanism of the stimulatory effects of light, were aimed along several different levels of integration. On the one hand, *in vitro* studies of CO_2-fixation by liquid cultures involving several plant generations led to the notion that a s.k.i. (succinate-ketoglutarate-isocitrate) cycle was operative during light-stimulated growth and CO_2 fixation whereby light, in some unknown fashion, mediated reductive carboxylation of ketoglutarate to isocitrate, the cleavage of isocitrate to succinate and a C_2 fragment, and perhaps the further conversion of part of the succinate back to ketoglutarate by a second reductive carboxylation. It was suggested that the C_2 piece (presumed to be glyoxylate) which resulted from cleavage of the isocitrate was involved in the increased proliferation induced by light. On the other hand, subsequent *in vivo* studies of the effect of light upon ontogeny led to the observations that the light effect was just as easily demonstrable when plants were grown for one generation only; parameters such as size of plants and time of discharge were directly related to the stimulatory effect of light (Cantino & Horenstein, 1957). Then it was determined (Cantino & Horenstein, 1959) that while the effect of light was completely dependent upon the presence of CO_2 or bicarbonate, the light and bicarbonate could be replaced with an equimolar mixture of succinate and glyoxylate, the presumed products of the reductive carboxylation of isocitrate in the S.K.I. cycle mentioned above. This provided substantial *in vivo* corroboration for a mechanism that had been derived from a more generally biochemical approach. In addition, our most recent studies (H. McCurdy & E. C. Cantino, to be published) have supplied new, direct evidence for the existence and role of the isocitritase and, even more pertinent here, a strong, pulling reaction subsequent to isocitritase; namely, a glyoxylate-alanine transaminase whose equilibrium lies in the direction of glycine and pyruvate and whose potential import for syntheses in general and light-stimulated growth in particular is obvious.

We had yet to establish, however—among other things—the exact locus for the light effect, the nature of the receptor, and the relationship between the formation of glyoxylate and glycine, on the one hand, and the biosynthetic reactions leading to increased synthesis of cytoplasmic and nuclear components, on the other. It was the purpose of this investigation to initiate an attack upon this latter, terminal area, and to determine if the light effect was reflected in demonstrable, intracellular changes during the very first few hours of ontogeny—that is, during spore germination and the subsequent formation of germlings.

MATERIALS AND METHODS

We have used the strain of *Blastocladiella emersonii* originally described by Cantino & Hyatt (1953). The media employed were a peptone-yeast–glucose medium PYG (Cantino, 1952), now commercially available as Difco Cantino PYG broth and agar, and a diluted version (see context) of the synthetic medium of Barner & Cantino (1952). Swarmer ('spore') suspensions were obtained by growing several thousand ordinary-colourless (O.C.) plants to maturity on PYG-agar, flooding the plates with sterile water when the population of first-generation plants was just beginning to discharge swarmers, and then collecting the swarmer suspension aseptically.

Nuclear counts were made on randomly selected germlings which were fixed with 10% formaldehyde and stained with 1:10,000 (w/v) toluidine blue, whereby the nuclei are clearly delimited and easily counted with an oil-immersion objective by virtue of their deeply stained, blue-violet central body against the faintly blue background of the nucleoplasm (Turian & Cantino, 1959). Spores were dried to constant temperature at 99° for dry-weight determinations. Ultraviolet absorption data were obtained in cells of 1 cm. light path with a model DU Beckman spectrophotometer. For most studies, white light (fluorescent) was used at a level of 390 f.c.; for the work at 29°, however, it was about 300 f.c. Aminopterin and pteropterin were very kindly supplied by Dr J. M. Ruegsegger of the Lederle Laboratories. Purines and pyrimidines were obtained from Sigma Chemical Company.

The methods of McIntire & Smith (1958), and McIntire & Sproull (1957) were adopted, with slight modifications to suit our particular problem, for the routine determination of DNA and total purines and pyrimidines (TPP). Individual bases in nucleic acid hydrolysates were separated on unwashed Whatman paper no. 1 by chromatography with water + saturated butanol, isolated by elution and determined spectrophotometrically (Hotchkiss, 1948). Analyses for orthophosphate and total phosphorus were made essentially according to the Fiske-Subbarow procedure outlined by Umbreit, Burris & Stauffer (1957). Total nitrogen was obtained by sulphuric acid digestions, subsequent nesslerization, and determinations with a Klett–Summerson colorimeter (cf. Umbreit *et al.* 1957).

RESULTS

In order to study the effect of light upon the initial stages of ontogeny, it was desirable to germinate simultaneously large quantities of spores and to synchronize subsequent development of the germlings for at least several hours. Actually, microscopic analyses of suspensions of germinating spores revealed that the degree of synchronization was already reasonably good without special treatments. However, attempts were made to improve it; the few positive results obtained are summarized below.

Indoleacetic acid (IAA)

This compound does affect the rate of spore germination, as it did in the close relative, Allomyces (Turian, 1956). When populations of spores were bathed in 2×10^{-5}M-indoleacetic acid at 26° for 30, 45 and 60 min. and then scored immediately for germination (along with untreated spores incubated for similar periods), it was found that indoleacetic acid did increase germination rates significantly, although the stimulatory effect decreased progressively with increased duration of treatment (Table 1). However, it did not improve the degree of synchronization over that of the control populations.

Heat shocks

Preliminary trials with different temperatures and time intervals revealed that a heat shock at 37° did improve synchronization. For example, spores were treated at this temperature for 5, 15, 30 and 60 min., they were then placed at 26° for 6 hr., and at this point we tabulated for each population the frequency of thalli in each of three arbitrary classes: ungerminated spores, small germlings (2–4 nuclei), and large germlings (over 4 nuclei). Maximum synchronization occurred with a 15 min. heat shock (Table 2).

Table 1. *Percentage increase in germination of spores induced by* 2×10^{-5} M *indoleacetic acid in* 1% *glucose solution at* 26°

Spore populations were fixed with 5% formaldehyde and scored at the times indicated below; the criterion of germination was the formation of the first, detectable rhizoidal system.

Duration of acid treatment from zero time (min.)	Percentage increase over controls without acid treatment
30	44
45	34
60	23
90	7
120	*c.* 0

Table 2. *The effect of heat shocks upon synchronization of germination*

Following the heat shocks, all populations were transferred to 26° and scored after 6 hr. of incubation.

Duration of initial heat shock at 37° (min.)	Percentage motile and resting, non-germinated spores	Percentage small germlings (2–4 nuclei)	Percentage large germlings (over 4 nuclei)
0	6	15	79
5	5	10	85
15	**3**	**6**	**91**
30	8	20	72
60	21	38	41

The effect of light on nuclear reproduction

Spores were germinated at 24° in liquid medium PYG and incubated 2, 3, 4, 5 and 6 hr. in the presence and absence of 390 f.c. of white light. The number of nuclei per plant was determined by direct, microscopic examination (Fig. 1). The number of nuclei per germling was decidedly greater in the light than in the dark for all incubation periods. Furthermore, when germlings incubated in light and dark were exposed simultaneously to thymidine and to uracil (10^{-4}): (*a*) uracil had no significant effect upon nuclear counts in either light or dark; (*b*) thymidine had no effect upon nuclear counts in the light; but (*c*) thymidine (and thymine) had a pronounced effect upon nuclear counts in the dark. Apparently, thymidine effectively stimulated nuclear multiplication to the same extent that light had done so! The progressive effect of light on the early ontogeny of *Blastocladiella emersonii* is clearly evident from the data in Fig. 2 in which we have averaged our total counts of nuclei in some hundreds of germlings grown for different times in light and dark. The inset in the figure delineates the clear-cut increase in nuclear reproduction brought about by light during the first 6 hr. of development. Finally, quite aside from the light effect itself, the abrupt change in slope of the curves at about 4 hr. is also worthy of note.

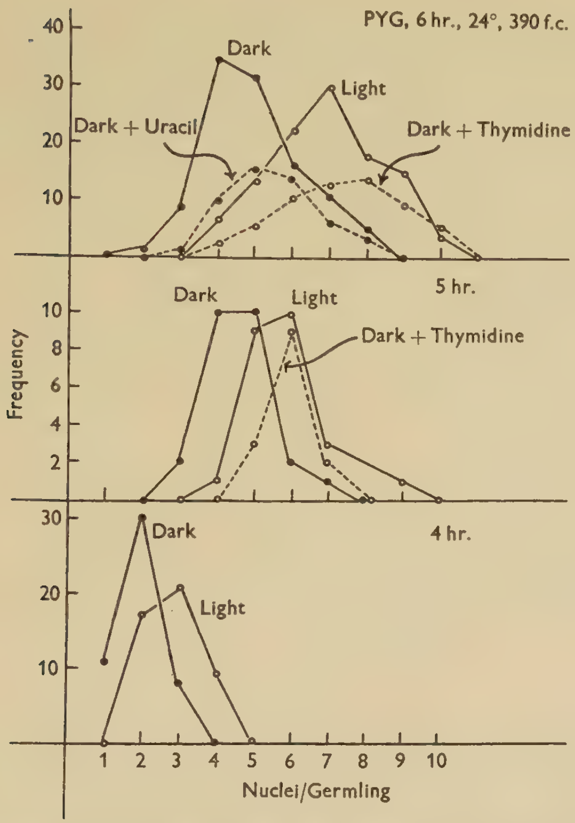

Fig. 1. The distribution curves for the number of nuclei found in young germlings grown for 6, 5 and 4 hr. on medium PYG at 24° in the presence and absence of 390 f.c. of white light and with and without incorporation of uracil and thymine (and thymidine) in the medium. At these ages germlings were all of equal size.

The effect of light upon nucleic acid synthesis

The methods of McIntire & Smith (1958), and McIntire & Sproull (1957) were selected for the routine estimation of the total DNA and RNA content of germinating spores and germlings in our comparative studies because of their speed and simplicity. Actually, the estimation of RNA is based upon a measure of total purines and pyrimidines (TPP), on the one hand, and the isolation of DNA and its subsequent analysis in a separate fraction, on the other, the difference being referred to as RNA in our work. In effect, the RNA value is really a measure of RNA plus a small, soluble pool of purines and pyrimidines present in *Blastocladiella emersonii.*

In any event, the ratio of DNA to TPP (an approximate ratio of DNA to total nucleic acid) was determined during germination and growth of spores for one generation; the data revealed (Fig. 3) that this ratio was greater in light-grown plants of different ages than it was in comparable dark-grown plants. Thus, it seems as if DNA increases more rapidly than RNA in light-grown plants than it does in dark-grown plants; this corresponds nicely with the observed increases in nuclear reproduction brought about by light during the early stages of germination and growth. In the close relative, Allomyces, the % DNA of total nucleic acids is also increased (*c.* 25 %) by providing the sexual (gametophytic) generation of the fungus with thymine. This observation may have an important bearing on the discovery (Turian, 1958) that thymine induces increased differentiation of male sex organs in Allomyces (cf. Cantino & Turian, 1959, for discussion).

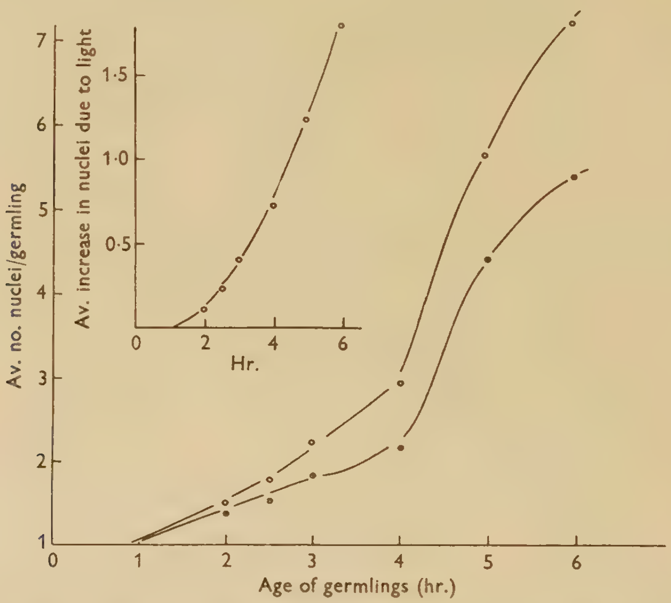

Fig. 2. The average number of nuclei in germlings grown for 2, 3, 4, 5 and 6 hr. on medium PYG at 24° in the presence and absence of 390 f.c. of white light.

The effect of analogues and inhibitors on nucleic acid synthesis

Hoping that it would further help us to understand the nature of the changes brought about by light, the effects of a few, selected analogues and potential inhibitors of nucleic acid metabolism, were also tested. In this case, 1/20 strength medium PYG was used in order to reduce the concentration of nucleic acid components present in the yeast extract which might have functioned as antagonists for the analogues employed. The pertinent results were as follows: while bromouracil and pteropterin had only rather small effects upon the DNA/TPP ratio, isocytosine, thiouracil and aminopterin did induce a striking change; the latter three brought about 50–100 % increases in the DNA/TPP ratio (Table 3).

Finally, absolute values for DNA and RNA (defined as TPP less DNA) were related to one another per unit weight of spores during the germination. For this purpose, replicate samples of spore suspensions used for analyses were sacrificed for the dry-weight determinations. Depending upon the batch of spores used, the initial concentration of DNA in the ungerminated spores varied between 13 and 17 μg DNA/mg. dry weight (average, 14·8); that of RNA varied between 190 and 270 μg RNA/mg. dry weight (average, 217). Once the young germlings were analysed, however, it became abundantly clear that during the early stages of spore germination, the increase in DNA/TPP which occurred (Fig. 3) was due to a pronounced increase in DNA synthesis while the concentration of RNA per cell changed very little (Fig. 4).

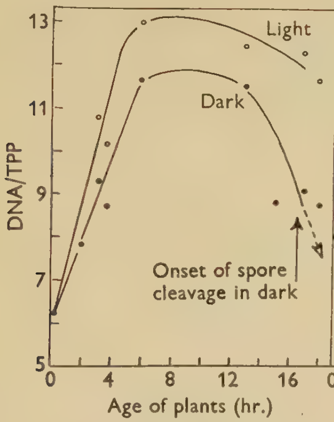

Fig. 3

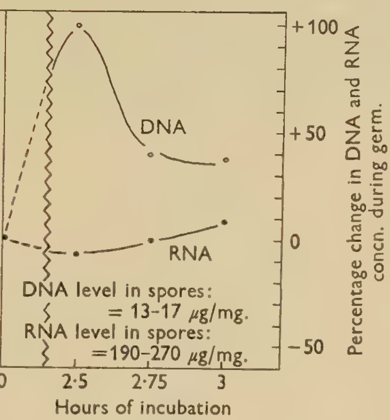

Fig. 4

Fig. 3. The percentage DNA/TPP (total purines and pyrimidines) throughout ontogeny in light (390 f.c.) and dark. TPP in light and dark were the same.

Fig. 4. The percentage change in concentration of DNA and RNA in spores during a 3 hr. germination period in the dark. (The same relationship is obtained from pentose analyses, using orcinol (Umbreit, 1957), although absolute values average 20 % low.)

Table 3. *The effect of various compounds upon the DNA/TPP ratio in germlings of* Blastocladiella emersonii

Spores were germinated in medium PYG. 1/20 strength, in the presence and absence of 10^{-3}M substrates for the times specified at 24° in the dark. The data are expressed as percentage change of the DNA/TPP ratio from that of control cultures on the PYG medium lacking added substrates.

Age of germlings at time of analysis (hr.)	Compounds incorporated in medium PYG				
	Bromouracil	Thiouracil	Pteropterin	Isocytosine	Aminopterin
	Percentage change in the DNA/TPP ratio				
5	− 2	.	− 8	+80	.
6	.	+105	−18	.	+95
7	+20	.	.	+57	+75

The composition of the DNA in Blastocladiella emersonii

The DNA isolated from light- and dark-grown, synchronized cultures (14 hr. plants) by the salamine methods previously described was subjected to hydrolysis with 2·4 N-HCl for 2 hr. (in an autoclave at 120°, 15 lb. pressure) and chromatographed in water-saturated butanol according to Hotchkiss (1948), along with authentic samples of the four principal DNA bases. The results (Fig. 5) revealed that: (*a*) the DNA contained the expected bases thymine, adenine, and cytosine (and probably guanine, whose mobility is low in the solvent system used); (*b*) the DNA seemed to contain two, unidentified components which peak in the region of 260 mμ (the possibility that they are simply incomplete-hydrolysis products has not been ruled out); and (*c*) no clearly significant differences between DNA from light-grown plants and DNA from dark-grown plants was detected, although the possibility remains that the ratio of the individual bases to one another may be affected by exposure of the plants to light. This possibility is to be pursued further.

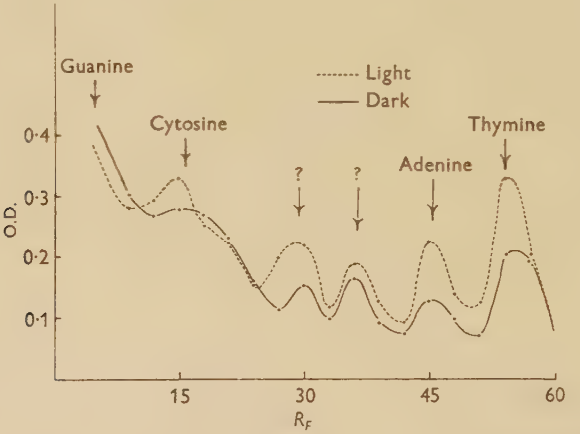

Fig. 5. The composition of HCl hydrolysates of DNA isolated from *Blastocladiella emersonii* as revealed by chromatography in water-saturated butanol according to Hotchkiss (1948) and measured at 260 mμ.

The effect of light upon phosphorus metabolism during germination and early development of germlings

On full-strength synthetic medium D (Barner & Cantino, 1952; containing glucose, glutamate, methionine, phosphate, magnesium sulphate and thiamine), the growth of *Blastocladiella emersonii* is also stimulated by light (Cantino, unpublished). Similarly, on this same medium the stimulatory effect of light upon nuclear multiplication was also demonstrated during our current studies. In the following experiments, spores were germinated in 1/10 strength medium D but with the glucose at 0·005 %.

Over the 4 hr. period during which spore germination and growth of germlings was followed, there was no net uptake of phosphate; indeed, there was apparently a net release, the orthophosphate pool increasing more in the light than in the dark (Fig. 6, top). On the other hand, the extractable (TCA-soluble) nitrogen pool increased much more rapidly in the light than in the dark (Fig. 6, centre). Thus, the relative pool of orthophosphate *per unit of TCA-soluble nitrogen* actually decreased much more rapidly in the light than it did in the dark during the early stages of spore germination (Fig. 6, bottom). Furthermore, the absolute pool of total TCA-soluble phosphorus also decreased more rapidly in the light than it did in the dark (Fig. 7). Apparently, this light-stimulated decrease in the soluble organic phosphorus was due, in the main, to a sharp decrease in the barium-insoluble fraction (B.I.F.) and the barium-soluble-alcohol-insoluble (B.S.A.I.F.) fractions of the TCA-soluble phosphorus pool (Fig. 7).

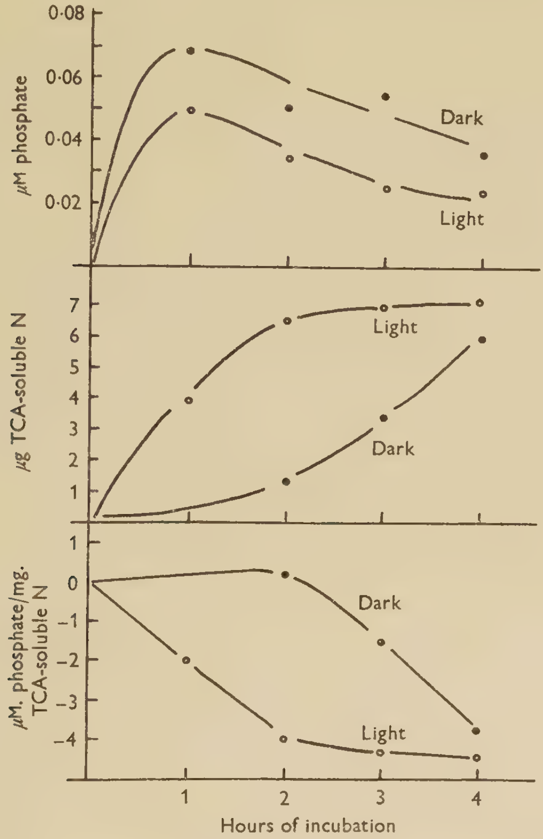

Fig. 6. Changes in the nitrogen and phosphorus pool in *Blastocladiella emersonii* during a 4 hr. germination period at 29° in light (390 f.c.) and dark on 1/10 strength medium D containing 0·005 % glucose. (Top): formation and re-utilization of orthophosphate; (centre): extractable TCA-soluble nitrogen; (bottom): the relative pool of orthophosphate per unit TCA-soluble nitrogen.

DISCUSSION

Gradually, a picture (but, admittedly, still a fuzzy one) of the stimulatory effect of light upon the growth of *Blastocladiella emersonii* is beginning to take shape. At this stage in the evolution of our perception of the situation, perhaps we can best visualize the picture as one with two focal points—a posterior and an anterior end, so to speak—connected by a thread of unknown magnitude and complexity (cf. Fig. 8).

At one end of this picture lies a block of reactions in which reductive carboxylation of ketoglutarate (via a TPN-specific isocitric dehydrogenase) provides the substrate for a subsequent reaction in the glyoxylate cycle (e.g. Kornberg & Krebs, 1957)—namely, cleavage of isocitrate to succinate and glyoxylate (Cantino & Horenstein, 1956, 1957, 1959; H. McCurdy & E. C. Cantino, to be published). In *Blastocladiella emersonii* the equilibrium of a transamination between glyoxylate and alanine to yield pyruvate and glycine is such as to constitute a potentially strong, pulling reaction (H. McCurdy &

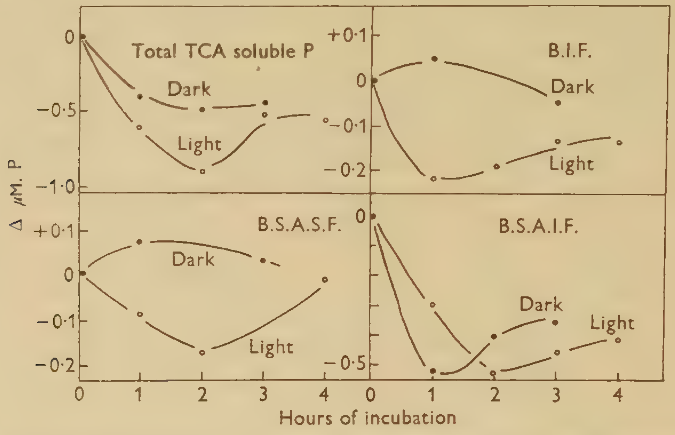

Fig. 7. Changes in the absolute pool of TCA-soluble phosphorus in *Blastocladiella emersonii* during a 4 hr. germination period at 29° in light (390 f.c.) and dark on 1/10 strength medium D containing 0·005 % glucose. (Upper left): total TCA-soluble P; (upper right): barium-insoluble-fraction of the total TCA-soluble P; (lower left): barium-soluble-alcohol-insoluble fraction of the total TCA-soluble P; (lower right): barium-soluble-alcohol-soluble fraction of the total TCA-soluble P.

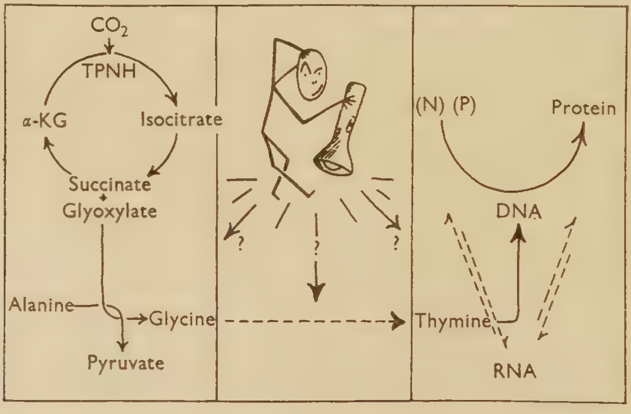

Fig. 8. Schematic digest of our current state of knowledge of light-stimulated growth in *Blastocladiella emersonii* (see text for explanation).

E. C. Cantino, to be published) for the isocitritase reaction and, therefore, for the reductive carboxylation of ketoglutarate (provided, of course, that reduced TPN is made available). Light increases the rate at which CO_2 is fixed via ketoglutarate reduction. It is not at all clear how this effect of light is exerted—whether it 'pushes' the reaction by production of increased reducing power to drive the carboxylation, 'pulls' the reaction by accelerating some step subsequent to the carboxylation, or causes some other effect. But the fact remains that CO_2 or bicarbonate is essential for the light effect, and that one can substitute for radiant energy the products of the reaction which it seems to accelerate—that is, an equimolar mixture of glyoxylate and succinate (Cantino & Horenstein, 1959).

At the other end lies the second block of reactions, and this region of metabolism also proceeds in higher gear when light is present; the visible, end result is an accelerated rate of nuclear reproduction. (Judging from reports such as those of Ogata, Shimizu & Togashi, 1958; Stegwee, 1955; Caspersson, 1950; Brachet, 1957, etc., this could mean increased rates of protein synthesis as well.) Furthermore, this light-stimulated increase in microscopically detectable nuclear synthesis is paralleled by a light-stimulated increase in the ratio of DNA to total nucleic acids as determined by chemical analysis. But here, too, the stimulatory effect of light can be replaced by supplementing the organism with a nutrient, in this case, thymine! Perhaps, then, biosynthesis of thymine is a natural, limiting factor in the growth of *Blastocladiella emersonii*, and supplementation of the thymine pool (by addition of thymine in the dark, or by a light-induced, increased synthesis) overcomes the barrier.

In any event, we are led to the interesting conclusion that either glyoxylate plus succinate, on the one hand, or thymine on the other, can substitute for light in stimulating growth. The literature dealing with the role of amino acids in nucleic acid synthesis is expanding rapidly; for glycine, in particular, the evidence suggests that it is involved in nucleic acid synthesis (e.g. Crosbie, 1958; Sutton, Schlenk & Werkman, 1951; Kritskii & Myagkaya, 1956, and others). In view of the aforementioned presence of a strong glyoxylate-alanine transaminase in *Blastocladiella emersonii* (H. McCurdy & E. C. Cantino, to be published) which leads to the formation of glycine, we have selected the working hypothesis that light, in some unknown fashion, accelerates the following chain of events: reductive carboxylation of ketoglutarate to yield isocitrate, cleavage of isocitrate to succinate and glyoxylate, transamination of glyoxylate with alanine to yield glycine, conversion of glycine to thymine, and, finally, metabolism of thymine to yield DNA and, therefore, increased protein synthesis and increased growth (Fig. 8).

Obviously, the principal hurdle to be overcome at the biochemical level is the nature of the link between the posterior and anterior ends of this picture—and at the biophysical level, the locus for the stimulatory effect of light and the mechanism of its action. But, whatever the nature of the final answer, this seems to be a unique phenomenon: that of a mould, without chlorophyll and without coloured carotenoids, which produces more nuclei and more DNA, fixes more CO_2, and grows more rapidly in the light than in the dark!

Apart from the photosensitivity of *Blastocladiella emersonii*, the nucleic acid transformations during spore germination deserve special attention. The blastocladiaceous spore is organized in intriguing fashion into intracellular compartments, one of the most obvious and most interesting being the RNA-rich nuclear cap (cf. Cantino & Turian, 1959, for discussion). During germination, the latter disorganizes and eventually disappears. Our determinations of DNA and TPP during germination may provide an important clue for understanding the reciprocal relationship between nuclear DNA and para-nuclear-nuclear RNA at this critical stage in ontogeny.

Our analytical data consistently revealed that the TPP (or approximately total nucleic acid) per cell remained essentially constant, from the zoospore stage to that of a young, 2½ hr. germling (Fig. 4). This steady level, however, implies a gradual decrease in nucleic acids per unit dry weight, for the latter increases between the first 1½–3 hr. following germination as synthesis of proteins, chitin, etc. accompanies the enlargement of the cell and the growth of rhizoids. Simultaneously, however, the DNA content per cell doubles just before the outset of the first mitosis (as it does, for example, in bacteria; Barner & Cohen, 1956) which occurs between 2 and 3 hr. after germination (Fig. 1). If, during this time, the total nucleic acid is constant, as it seems to be, one can hardly escape the conclusion that the sudden increase in DNA occurs at the expense of the total pool of TPP, as illustrated in Table 4.

Table 4. *Change in absolute value of nucleic acid components during germination, expressed in $\mu g/10^6$ cells (spores or germlings)*

	TPP	DNA	'RNA'*
Spores	403	28	375
2½ hr. germlings	408	53	355
Change	+5	+25	−20

* 'RNA' equals RNA plus the pool of nucleotides, nucleosides, purines and pyrimidines.

Moreover, the fact that this nucleic acid pool is probably concentrated in the main, in the RNA nuclear cap at the swarmer (zoospore) stage (Turian, 1955) suggests a function for this cytoplasmic body found in the Blastocladiaceae. Its essential role during germination, when it disintegrates and donates its basophilic constituents to the cytoplasm, would be associated not only with resumption of protein synthesis (Turian, 1956) but also with its capacity to serve as a reservoir of nucleic acid building blocks for the sudden synthesis of DNA in the nucleus. Thus, the doubling of the DNA which precedes the first mitosis corresponds to a sort of 'partial synthesis' of DNA from pre-existing nucleic acid precursors such as the nucleosides and nucleotides made available during the disintegration of the nuclear cap during germination.

Such a 'partial synthesis' of DNA from a pre-existing pool of nucleic acids and their building blocks is consistent with our earlier data (Turian, 1958) concerning: (*a*) the absence of dinitrophenol toxicity during the first stages of germination (up to the 3 hr. binucleate germling stage) in Allomyces; and (*b*) the strong, inhibitory effect of dinitrophenol upon further growth of germlings of Allomyces in which total synthesis of nucleic acids is subsequently presumed to occur (and, in *Blastocladiella emersonii*, does occur; see Fig. 3).

In this connexion, one last thought: it is axiomatic that parallel synthesis of both DNA and RNA must proceed during the development of young germlings into mature plants; presumably, this occurs at a steady state if the ratio of the two components is to be maintained at a temporarily constant level (Fig. 3). But, in diametric opposition to the situation that occurs during germination, it must be assumed that DNA synthesis stops earlier than RNA synthesis during ontogeny if the ratio of DNA/TPP is to decrease ultimately to *c.* 6 % when the mature plant once again undergoes progressive cleavage into swarmers containing the RNA-rich, nuclear caps.

Finally, the use of compounds antagonistic to nucleic acid metabolism has provided additional clues for an understanding of nucleic acid synthesis during germination in *Blastocladiella emersonii.* It was to be expected that analogues such as thiouracil and isocytosine would inhibit RNA synthesis (which is resumed some 3 hr. following germination; see Fig. 4) and thus increase the DNA/TPP ratio in *B. emersonii.* This was in fact the case, although thiouracil was effective only at rather high concentrations; at 2×10^{-4} M, it did not modify the DNA/TPP ratio. On the other hand, 5-bromouracil behaved more or less like thymine, and this was perhaps not unexpected in view of the substitution of this analogue for thymine in bacterial DNA (e.g. Zamenhof, Rich & de Giovanni, 1958; Cohen & Barner, 1956; Dunn & Smith, 1957).

With folic acid analogues, however, the results were particularly interesting, and somewhat unexpected. Pteropterin, a rather soluble compound, induced a decrease in the DNA/'RNA' ratio, presumably through a selectively inhibitory effect on DNA synthesis, at concentrations which did not inhibit growth. On the contrary, aminopterin (less soluble and more toxic) produced surprisingly high ratios, especially at growth-reducing concentrations. This was accompanied by striking cytological effects: elongated germlings with low basophily and only one or at most two or three giant nuclei (and nucleoli) in cytoplasmic compartments with space for six to eight normal-sized nuclei (cf. Nickerson & Mankowski, 1953; and Nickerson & Webb, 1956, for induced filamentation in Candida and *Bacillus cereus* by aminopterin). One explanation for the increased ratio, reinforced by the above cytopathological effects, would be that partial DNA synthesis from the nucleic acid pool is unaffected, and even proceeds faster than normal, while further nucleic acid synthesis from smaller building blocks is sharply inhibited. In this sense, then, the aminopterin effect would provide indirect confirmation of the notion that conversion of TPP to DNA is associated with the function of the nuclear cap as a reservoir of nucleic acids.

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A Comparison of Four Species of Mycobacteria

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SUMMARY: Expanded descriptions of *Mycobacterium phlei* Lehmann & Neumann, *M. smegmatis* (Trevisan) Lehmann & Neumann, *M. fortuitum* Cruz, and the species tentatively designated as *M. rhodochrous* (Overbeck) Gordon & Mihm are presented. These characterizations are based on 40, 95, 80, and 79 strains respectively, upon which 39 different tests and observations were made.

In 1951, a taxonomic study was begun of the nocardias, streptomycetes, and rapidly growing strains of mycobacteria. Subsequently, a goodly number of more slowly growing strains of mycobacteria was received, mainly from diagnostic laboratories. After varying periods of cultivation, some of these slowly growing strains grew as abundantly at 28° as those of *Mycobacterium phlei*, for example, and it became evident that separation of mycobacteria by their growth rate was both arbitrary and unfounded. In 1956, therefore, we included in this taxonomic investigation the so-called atypical, chromogenic, and other variously designated strains, the cause of increasing concern in the diagnostic laboratories. These strains were compared with those of the four species of mycobacteria described earlier, and stable, correlating criteria were sought for their specific identification. Thus far, this search has perversely yielded only additional means of recognizing strains of the species already characterized. As these additional criteria are especially useful for identifying strains of *M. fortuitum*, a species represented by more than 20 % of our strains of mycobacteria obtained from laboratories associated with human and veterinary medicine, the expanded descriptions are presented here.

METHODS

The strains of *Mycobacterium phlei*, *M. smegmatis*, *M. fortuitum*, and *M. rhodochrous* included those listed previously (Gordon & Smith, 1953, 1955; Gordon & Mihm, 1957) and those given in Tables 1–4.

Microscopic examination. The cultures were grown on glycerol agar (peptone, 5 g.; beef extract, 3 g.; glycerol, 70 ml.; agar, 15 g.; soil extract, 250 ml.; tap water, 750 ml.; pH 7·0) or on blood base agar (Difco) plus 7 % (v/v) glycerol for 5 days at 28° and stained by the Ziehl–Neelsen method.

As the strains of mycobacteria were compared with those of *Nocardia* spp. and *Streptomyces* spp., observations of colonial morphology were made on Bennett's agar (yeast extract, 1 g.; beef extract, 1 g.; N-Z Amine A (Sheffield Farms Inc., New York, N.Y.), 2 g.; glucose, 10 g.; agar, 15 g.; distilled water, 1000 ml.; pH 7·3) and on soil-extract agar (peptone, 5 g.; beef extract, 3 g.;

agar, 15 g.; soil extract, 250 ml.; tap water, 750 ml.; pH 7·0). One loopful of a 7- to 14-day culture in glucose broth was inoculated into tubes of Bennett's agar and soil-extract agar that had been melted and cooled to 45°. After careful mixing of the inoculum, approximately 1 ml. of each agar was pipetted on to a sterile microscope slide inside a sterile, moist chamber and incubated at 28°. The slides were examined (magnification, ×430) at 3, 7 and 14 days for the types of colony present. An alternative method was the pipetting of the inoculated agar into the depression of a sterile, hanging-drop slide inside a sterile, moist chamber, filling the depression $\frac{2}{3}$ to $\frac{3}{4}$ full. Before observation (magnification, ×100), the culture was covered with a cover slip, held in place with 'Vaseline'.

Table 1. *Additional strains identified as* Mycobacterium phlei *Lehmann & Neumann*

Laboratory no.	Name when received, source, and strain name or number
N525	*Mycobacterium phlei* Lehmann & Neumann; NCTC (525)
A11728	*M. phlei*; ATCC (11728); J. M. Desranleau
A11758a	*M. phlei*; ATCC (11758a); G. Penso
589	*M. phlei*; J. Glover, Univ. of Liverpool (Crottin); A. Lutz
594	*M. phlei*; J. W. Hornibrook, Nat. Inst. of Health, Bethesda, Md. (581)
811, 812	*M. phlei*; L. S. McClung, Indiana Univ. (X39, IUMC)
854, 855	*M. phlei*; E. Lederer, Inst. de Biologie Physico-Chimique, Paris (Fleole Pasteur, ICI)
N10,008	*Mycobacterium* sp.; NCTC (10,008, Kedrowsky's bacillus)
682–684	*Mycobacterium* spp.; E. R. Brown, USAF Hospital, Austin, Texas (2, syphilitic lesion; 3, sputum; 1, gastric washings)

Table 2. *Additional strains identified as* Mycobacterium smegmatis *(Trevisan) Lehmann & Neumann*

Laboratory no.	Name when received, source, and strain name or number
813, 814	*Mycobacterium avium* Chester; L. S. McClung, Indiana Univ. (X85, A)
A11314	*M. butyricum* Bergey *et al.*; ATCC (11314); F. Stimpert (04909)
582	*M. chelonei* Bergey *et al.*; J. W. Hornibrook, Nat. Inst. of Health, Bethesda, Md. (174)
A11759	*M. lacticola* Lehmann & Neumann; ATCC (11759); G. Penso (Milch)
580	*M. ranae* (Küster) Bergey *et al.*; J. Q. Heplar, Long Island Biological Assn., Cold Spring Harbor, N.Y. (Steenken)
675	*M. ranae*; E. N. Azarowicz, Univ. of Calif. (lymph node)
A11727	*M. smegmatis* (Trevisan) Lehmann & Neumann; ATCC (11727); J. M. Desranleau
672	*M. smegmatis*; A. Abrams, Walter Reed Army Medical Center (52-L-1)
853	*M. smegmatis*; E. Lederer, Inst. de Biologie Physico-Chimique, Paris
595	*M. stercoris* Bergey *et al.*; A. Abrams (52-Q-1)
519	*Mycobacterium* sp.; G. Pacheco, Inst. Oswaldo Cruz, Brazil (190, sputum)
592, 593	*Mycobacterium* spp.; J. W. Hornibrook (370, 430, associated with human leprosy)
661	*Mycobacterium* sp.; J. W. Hornibrook (366, associated with rat leprosy)
815	*Mycobacterium* sp.; L. S. McClung (X40)
831	Unidentified isolation; C. C. Campbell, Walter Reed Army Medical Center (573)

Decomposition of casein. Skim milk or skim-milk powder suspended in the required amount of water and an equal volume of 2 % (w/v) water agar were sterilized separately by autoclaving, cooled to 47°, mixed, and poured into sterile plates (5 plates from 100 ml.). Each culture was streaked on a plate and inspected for clearing of the casein after incubation at 28° for 7 and 14 days (Hastings, 1903).

Table 3. *Additional strains identified as* Mycobacterium fortuitum *Cruz*

Laboratory no.	Name when received, source, and strain name or number
N2006	*Mycobacterium fortuitum* Cruz; NCTC (2006)
662–666	*M. fortuitum*; S. McMillen, Hektoen Inst. for Medical Research (Tb-11, Tb-166, Tb-167, 266, 2338)
922	*M. fortuitum*; F. B. Seibert, Henry Phipps Inst. (P.H.S. 12); S. McMillen (12, sputum)
961–964	*M. fortuitum*; L. G. Wayne, Veterans Administration Hospital, San Fernando, Calif. (76, 212, 213, 214)
691–696	*M. minetti* Penso *et al.*; D. W. Will, Olive View Sanatorium, Olive View, Calif. (Goring, C18, C19, enteriditis, C70, C75); G. Penso
N2891	*M. ranae* (Küster) Bergey *et al.*; NCTC (2891); ATCC (110)
N3631	*M. tuberculosis* (Schroeter) Lehmann & Neumann; NCTC (3631, avian type from mesenteric glands of a cow)
N1542*	*Mycobacterium* sp.; NCTC (1542); Král Collection (fish)
12, 13	*Mycobacterium* spp.; R. E. Gordon, Dept. of Agric., Washington, D.C. (soil)
478	*Mycobacterium* sp.; P. Stuart, Ministry of Agric. and Fisheries, Weybridge, Surrey (Ms. 229.52)
518, 520–522	*Mycobacterium* spp.; G. Pacheco, Inst. Oswaldo Cruz (189, sputum; 191, gastric juice; 192, sputum; 193, urine)
640, 667, 723, 725	*Mycobacterium* spp.; D. Yegian, State Tuberculosis Hospital, Ray Brook, N.Y. (53112, 53111, 53108, 53109); M. E. Clark (sewage)
644–646, 726	*Mycobacterium* spp.; D. Yegian (53104, 53102, 53106, 5313); M. E. Clark (sputum)
681	*Mycobacterium* sp.; E. N. Azarowicz, Univ. of Calif. (M18)
720	*Mycobacterium* sp.; G. Altmann, Tel Hashomer Hospital, Israel (TH/2886, gastric juice)
786–788	*Mycobacterium* spp.; J. Ross, Univ. of Washington (Oak Ridge, Columbia River, Carson; fish)
789A, 789B	*Mycobacterium* spp.; J. Ross (variants of Chambers Creek; fish)
936, 937, 945	*Mycobacterium* spp.; U.S. Public Health Service, Muscogee County Health Dept., Ga. (P.H.S. 10, P.H.S. 11, P.H.S. 81; sputum)
965–967	*Mycobacterium* spp.; L. G. Wayne (80, 211, 313)
970	*Mycobacterium* sp.; D. H. Howard, Univ. of Calif. (134, skin ulcer)
981	*Mycobacterium* sp.; L. F. Affronti, Henry Phipps Inst. (P.H.S. 383); E. H. Runyon
734	Unidentified isolation; C. C. Campbell, Walter Reed Army Medical Center (931)
820	Unidentified isolation; O. Kanner, Veterans Administration Hospital, Oteen, N. C. (Hagans, sputum)

* Same as ATCC strain 9820, erroneously listed by Gordon & Smith (1955) as NCTC strain 2014.

Decomposition of tyrosine. Each culture was streaked once across a plate containing approximately 20 ml. of the following medium: peptone, 5 g.; beef extract, 3 g.; agar, 15 g.; tyrosine, 5 g.; distilled water, 1000 ml.; pH 7·0. An even distribution of the crystals of tyrosine was insured by placing 0·5 g.

of tyrosine in a 250 ml. flask, adding 100 ml. of the remaining components, autoclaving, cooling to 47°, and carefully mixing the agar before and during pouring. After 14 and 21 days of incubation at 28°, the plates were observed for the disappearance of the crystals underneath and bordering the growth.

Hydrolysis of starch. Duplicate plates of the following medium were streaked and incubated at 28°: peptone, 5 g.; beef extract, 3 g.; agar, 15 g.; potato starch, 10 g.; distilled water, 1000 ml.; pH 7·0. The starch was suspended in 50 ml. of cold water prior to its addition to the melted agar. Before the plates were poured, the agar was thoroughly mixed to distribute the starch evenly. One plate was flooded with 95 % ethanol at 5 days, and the other at 10 days. After 20–30 min., the unchanged starch became white and opaque, while a clear zone underneath and around the growth measured the hydrolysis of the starch (Kellerman & McBeth, 1912).

Table 4. *Additional strains identified as* Mycobacterium rhodochrous *(Overbeck) Gordon & Mihm*

Laboratory no.	Name when received, source, and strain name or number
804	*Erythrobacillus havaniensis*; L. S. McClung, Indiana Univ. (RH 181); R. S. Breed (4001)
802	*Mycobacterium agreste* Gray & Thornton; L. S. McClung (RH 176); R. S. Breed (2565)
801	*M. rubropertinctum* (Hefferan) Ford; L. S. McClung (RH 175); R. S. Breed (185)
816, 819	*Mycobacterium* spp.; L. S. McClung (B 161 B, 41 B)
568–570	*Nocardia* spp.; N. M. McClung, Univ. of Kansas (14, 15, 16; soil)
729	*Nocardia* sp.; L. Ajello, Communicable Disease Center, Chamblee, Ga. (M 172–6); W. M. Bowman.
764	*Nocardia* sp.; D. M. Webley, Macaulay Inst., Aberdeen (P_2); B. Treccani
822	*Nocardia* sp.; M. P. Lechevalier, Rutgers Univ. (24E, soil)
836, 838, 839	*Nocardia* spp.; J. B. Davis, Magnolia Petroleum Co., Dallas, Texas (18–68, 18–2, 107–308; utilize ethane, Davis, Chase & Raymond, 1956)
A 12483	*Nocardia* sp.; ATCC (12483); Amer. Cyanamid Co.
766	*Proactinomyces caprae*; D. M. Webley (T_3)
765 A, 765 B	*P. opacus* (den Dooren de Jong) Jensen; D. M. Webley (variants of T_1)
759, 760	*P. ruber* Krassilnikov; D. M. Webley
805, 809	Unidentified isolations; L. S. McClung (RH 185, RH 210); R. S. Breed (B 17, GLF)
1022	Unidentified isolation; C. C. Campbell, Walter Reed Army Medical Center (188, resected lung tissue)

Temperatures of growth. Cultures were made on slants of glycerol agar, heated or cooled to the desired temperature in a water bath, then transferred to a water bath at the same temperature inside a constant temperature incubator. The water level and temperature of the water bath were carefully watched. The cultures were examined for growth after 5–7 days at temperatures of 35° or above, and after 3 weeks at 10°.

Survival at 60°. Slants of glycerol agar were inoculated, quickly heated to 60° in a water bath, then placed in another water bath at 60° inside a constant temperature incubator. After 4 hr., the cultures were rapidly cooled, incubated at 28° for 14 days, and inspected for growth.

Acid production from carbohydrates. One-half ml. of a 10% (w/v) aqueous solution of each carbohydrate, sterilized by autoclaving, was pipetted aseptically to 5 ml. of sterile, inorganic nitrogen agar base: $(NH_4)_2HPO_4$, 1 g.; KCl, 0·2 g.; $MgSO_4.7H_2O$, 0·2 g.; agar, 15 g.; distilled water, 1000 ml. (Ayers, Rupp & Johnson, 1919). The pH value of this medium was adjusted to 7·0 before the addition of 15 ml. of a 0·04% (w/v) solution of bromcresol purple. Cultures on each carbohydrate agar were observed for the acid colour of the indicator after 7 and 28 days of incubation at 28°.

Utilization of organic acids as carbon sources. Modifications of Koser's (1924) citrate agar were prepared by adding 2 g. of Na benzoate, Na citrate, Na lactate, Ca malate, mucic acid, Na oxalate, or Na succinate to NaCl, 1 g.; $MgSO_4.7H_2O$, 0·2 g.; $(NH_4)_2HPO_4$, 1 g.; KH_2PO_4, 0·5 g.; agar, 15 g.; distilled water, 1000 ml.; 0·04% (w/v) solution of phenol red, 20 ml. To insure a slightly acid colour of the indicator in the sterilized agar, the pH value was adjusted before autoclaving as follows: benzoate, 7·0; citrate, 6·8; lactate, 6·8; malate, 7·2; mucate, 6·8; oxalate, 6·7; and succinate, 6·8. Use of the inorganic acid was indicated by the alkaline colour of the phenol red after 4 weeks of incubation at 28°.

Production of urease. Ten ml. of a 15% (w/v) solution of urea, sterilized by filtration, was combined with 75 ml. of the following autoclaved broth: KH_2PO_4, 9·1 g.; Na_2HPO_4, 9·5 g.; yeast extract, 0·1 g.; phenol red, 0·01 g.; distilled water, 1000 ml.; pH 6·8 (Rustigian & Stuart, 1941). The mixture was pipetted aseptically to sterile plugged test tubes in 1·5 ml. amounts and heavily inoculated with actively growing, 2- or 3-day-old cultures. Urease was demonstrated by the alkaline reaction of the phenol red after 5, 7, 14, 21, and 28 days at 28°.

Sensitivity to dyes. Malachite green oxalate, methyl violet, and pyronin B agars were made by adding 0·01% (w/v) of each dye to glycerol agar. Slants of each of the three agars and one of glycerol agar as a control were inoculated from a 7- to 14-day-old culture in glucose broth with a loop, 2·5 mm. in outside diameter. After incubation at 28° for 28 days, growth on the agars was recorded.

MacConkey agar. 7- to 14-day-old cultures in glucose broth were streaked on a slant of MacConkey agar (Difco) and on a control slant of glycerol agar with a loop, 2·5 mm. in outside diameter. Growth and colour change of the neutral red indicator were noted after 7 and 28 days at 28°.

RESULTS

Mycobacterium phlei *Lehmann & Neumann*

The 13 strains of this species added to the previously listed 27 (Gordon & Smith, 1953, 1955) were initially labelled *Mycobacterium phlei* or *Mycobacterium* spp. As misnamed strains or strains with specific names in synonymy were not received, annoying problems of nomenclature were eliminated.

When stained by the Ziehl–Neelsen method, cultures of the 13 strains showed coccobacilli; small, short rods; or small, pleomorphic rods. Forty to 95% of the rods retained the carbol fuchsin. Each of the 13 strains formed three or more of the following types of colonies: dense colonies with smooth edges; dense colonies with a halo or outcroppings of filaments; densely or loosely filamentous colonies; and thin, flat colonies of cells, sometimes resembling a medusa-head. Cultures growing in the smooth stage showed dense colonies with smooth edges, dense colonies with a fringe of short filaments, and tiny, filamentous colonies. Cultures growing in the rough stage had few, if any, dense colonies with smooth edges, and their filaments were longer and more extensive. During the 14 days of observation, the filaments of 10 of the 13 cultures fragmented into shorter forms.

The macroscopic appearance and physiological reactions of the 13 new strains did not differ from those of the 27 previously studied, and the results of the tests applied to the 40 strains of this species are presented in Table 5. The most important reactions for separating strains of *Mycobacterium phlei* from those of *M. smegmatis*, *M. fortuitum*, and *M. rhodochrous* were: growth at 52°; lack of growth at 10°; survival at 60° for 4 hr.; acid production from arabinose and sorbitol; failure to form acid from dulcitol, inositol, or rhamnose; inability to utilize benzoate, mucate, or oxalate; inhibition of growth by a 0·01% (w/v) concentration of malachite green, methyl violet, or pyronin; and lack of growth on MacConkey agar. Although the following properties had no value in distinguishing strains of *M. phlei* from those of the other three species, they were considered part of the pattern of reactions of this species: failure to decompose casein or tyrosine; hydrolysis of starch; acid formation from mannitol and mannose; inability to produce acid from lactose, raffinose, or α-methyl-D-glucoside; use of citrate, lactate, malate, and succinate as sources of carbon; and development of urease. Variation by a strain in one or two respects from the pattern of these 28 more dependable reactions did not prevent its identification as *M. phlei*.

Mycobacterium smegmatis (*Trevisan*) *Lehmann & Neumann*

Mycobacterium chelonei Bergey *et al.* (1923) was the only specific name among those of the 17 strains listed in Table 2 that had not been represented by one or more of the 78 strains previously identified as strains of *M. smegmatis*. The original description of *M. chelonei*, Friedmann's (1903) turtle bacillus, was based mainly on variable characteristics, but did not definitely challenge the authenticity of strain no. 582. The name *M. chelonei* was, therefore, accepted in the synonymy of *M. smegmatis*.

Two of the 17 strains were initially labelled *Mycobacterium ranae* (Küster) (Bergey *et al.* 1923). The original account of this species (Küster, 1905) described the first strain as unable to grow at 37°. As growth at 45° was a very stable characteristic of strains of *M. smegmatis*, Küster's strain was not believed to have been *M. smegmatis*. These two strains and seven others previously listed, received as *M. ranae* from laboratories in the U.S., were regarded as misnamed and not representative of *M. ranae*.

Eight cultures of the 17 were composed of pleomorphic rods, often unevenly stained by the Ziehl–Neelsen technique. The rest of the cultures were slender rods of short or medium length. In the 17 cultures, 25–100 % of the rods retained the carbol fuchsin. Each culture showed two or more of the following

Table 5. *Comparison of physiological characteristics*

Property	40 strains of *M. phlei* (% positive strains)	95 strains of *M. smegmatis* (% positive strains)	80 strains of *M. fortuitum* (% positive strains)	79 strains of *M. rhodochrous* (% positive strains)
Decomposition of				
Casein	0	0	0	0
Tyrosine	0	0	0	76
Hydrolysis of starch	100	100	100	97
Growth at				
52°	100	0	0	3
45°	100	100	15	54
40°	100	100	70	76
35°	100	100	95	90
28°	100	100	100	100
10°	0	44	46	100
Survival at 60°, 4 hr.	90	0	0	22
Acid from				
Arabinose	98	100	3	0
Dulcitol	0	98	0	0
Erythritol	52	100	18	4
Galactose	73	99	10	5
Glucose	91	100	100	97
Inositol	0	100	9	16
Lactose	0	0	0	0
Mannitol	100	98	26	100
Mannose	94	100	100	100
α-methyl-D-glucoside	0	0	0	0
Raffinose	0	0	1	0
Rhamnose	0	100	0	8
Sorbitol	100	100	3	100
Trehalose	73	99	100	96
Xylose	64	99	5	17
Utilization of				
Benzoate	0	95	3	80
Citrate	94	99	95	87
Lactate	100	100	100	99
Malate	100	100	100	99
Mucate	0	100	0	0
Oxalate	0	99	0	0
Succinate	97	100	99	100
Production of urease	98	100	96	75
Growth on dyes				
Malachite green	3	100	100	1
Methyl violet	0	99	100	0
Pyronin	5	96	100	0
Colour change of MacConkey agar	0	43	96	0

colonial types: dense colonies with smooth edges; dense colonies with a fringe or outcroppings of filaments; dense colonies with a halo of cells; loosely filamentous colonies; and very thin colonies of rods, often resembling a medusa-head. During incubation, the filaments of 11 cultures fragmented into short rods.

The growth and physiological characteristics of these 17 strains were similar to those of the 78 previously assigned to *Mycobacterium smegmatis*. The physiological reactions of the 95 strains of this species are given in Table 5. Distinctive properties were: growth at 45° but none at 52°; failure to survive at 60° for 4 hr.; production of acid from arabinose, dulcitol, erythritol, inositol, rhamnose, sorbitol, and xylose; utilization of benzoate, mucate, and oxalate; and growth on agar with 0·01 % (w/v) malachite green, methyl violet, or pyronin. Other criteria comprising the pattern of reactions of this species were: failure to decompose casein or tyrosine; hydrolysis of starch; acid production from galactose, glucose, mannitol, mannose, and trehalose; failure to form acid from lactose, α-methyl-D-glucoside, or raffinose; use of citrate, lactate, malate, and succinate; and the presence of urease.

Among all the mycobacteria in this collection (those in Table 5 plus 205 specifically unassigned strains) utilization of mucate and oxalate was peculiar to strains of *Mycobacterium smegmatis*.

Mycobacterium fortuitum *Cruz*

The nomenclature of this species is still unsettled. In 1955, Gordon & Smith found it difficult to believe that a strain of such a widely distributed species (soil, water, lesions in man, cattle, and cold-blooded animals) had not been described and named long before *Mycobacterium fortuitum* Cruz (1938). Had Cowan's (1956) proposal of the use of a common name for a species, whose scientific name was not established, been available, it would have been seriously considered.

In 1958, NCTC strain no. 2891 of *Mycobacterium ranae* was added to this collection. This strain was received by the NCTC many years ago from the ATCC as strain no. 110. Unlike the present ATCC strain no. 110 and eight other misnamed ones initially labelled *M. ranae* from laboratories in the U.S., NCTC no. 2891 was a strain of *M. fortuitum* and agreed more closely with Küster's first description of *M. ranae* (1905). Küster stated that his strain did not grow at 37°; NCTC strain no. 2891 grew at 40°, but not at 45°. The maximum temperatures of growth of the 80 strains representing *M. fortuitum* in this collection, however, varied considerably. There were four, isolated from fish, that did not grow at 35°; 19 others that did not grow at 40°; and 12 that grew slightly at 45°. After a few more years of cultivation *in vitro*, the 23 strains that did not grow at 40° will be tested again. If a substantial number of them, including the four from fish, grow at 40°, variation of the maximum temperature of growth of NCTC strain no. 2891 will be accepted, and the name *M. ranae* will be considered in the nomenclature of this species.

Mycobacterium minetti Penso *et al.*, typified by strains nos. 691 to 696, was previously assigned to the synonymy of *M. fortuitum*. According to our records, NCTC strain no. 3631, initially labelled *M. tuberculosis* avian type, was one of the four NCTC strains identified as *M. minetti* by Penso *et al.* (1952).

Thirty-five of the 52 strains of *Mycobacterium fortuitum* acquired since 1955 exhibited thin, very short to long rods, and the remaining 17 were composed of pleomorphic rods. The rods of 27 strains showed 90% or more retention of the carbol fuchsin; the rods of 10 strains, 50–85%; and those of 15 strains, 5–45%. The colonial morphology of the 52 strains was similar to that described for *M. smegmatis*. A strain growing in the smooth stage produced a preponderance of dense colonies as illustrated in Pl. 1, fig. 1. Thin colonies of rods (Pl. 1, fig. 2) were also formed. Most of the colonies of a strain growing in the rough stage, on the other hand, were loosely filamentous, and their filaments were fairly extensive (Pl. 1, figs. 3, 4). The filaments of all but 12 strains fragmented into shorter forms.

The growth of cultures of *Mycobacterium fortuitum* on glycerol agar was off-white or cream-coloured, soft and butyrous, or waxy and nodular. Occasionally an old culture on glycerol agar formed a black soluble pigment, as dark as the pigment of cultures of *Bacillus subtilis* var. *niger* on tyrosine agar. When first observed, these pigmented cultures were replated, but contaminants were not found. The physiological characters of the 80 strains assigned to this species are listed in Table 5. The most useful reactions for recognizing strains of *M. fortuitum* were: scant growth, if any, at 45°; lack of growth after 4 hr. at 60°; inability to form acid from arabinose, dulcitol, rhamnose, sorbitol, or xylose; failure to utilize benzoate, mucate, or oxalate; growth on malachite green, methyl violet, and pyronin agars; and growth on MacConkey agar changing the agar from deep fuchsia to yellowish pink or yellow. Other characteristics of this species were: inability to attack casein or tyrosine; hydrolysis of starch; demonstration of acid from glucose, mannose, and trehalose; failure to form acid from α-methyl-D-glucoside, lactose, or raffinose; use of citrate, lactate, malate, and succinate; and production of urease.

The 205 specifically unassigned strains of mycobacteria in this collection were also inoculated on malachite green, methyl violet, and pyronin agars. As some strains grew on one, two, or three of these agars, resistance to the three dyes was not limited to strains of *Mycobacterium fortuitum* and *M. smegmatis*. When streaked on MacConkey agar, the 205 strains grew slightly, if at all, and did not change the colour of the indicator during 6 weeks of incubation. A colour change of MacConkey agar was, therefore, peculiar to the strains of *M. fortuitum* and 43% of those of *M. smegmatis*.

Mycobacterium rhodochrous (*Overbeck*) *Gordon & Mihm*

This taxon has often been described in this laboratory as a good species in search of a genus. Its strains in this collection have been placed by different investigators in the following genera: *Bacillus*, *Bacterium*, *Erythrobacillus*, *Micrococcus*, *Mycobacterium*, *Nocardia*, *Proactinomyces*, *Rhodococcus*, and *Serratia*. Although strongly tempted to assign this species to the genus *Corynebacterium*, Gordon & Mihm (1957) would not do so without a comparative study of the corynebacteria. It was tentatively allotted, therefore, to the genus *Mycobacterium* because the colonies of 54 of the 56 strains of this species were like those of mycobacteria. Colonies of strains of *M. rhodochrous* growing in the smooth state (Pl. 2, figs. 5, 6) resembled those of smooth strains of mycobacteria (Pl. 1, figs. 1, 2), rather than colonies of nocardias as typified by *Nocardia asteroides* (*N. farcinica*). Acid-fastness was also considered. Many strains of this species, of which only nos. 381, 382, and 386 remain, were isolated during a study of the acid-fast bacteria in soil (Gordon & Hagan, 1937). When first examined, they were as strongly acid-fast as strains of *M. phlei* or *M. smegmatis*. After a few transfers, however, most of them no longer retained the carbol fuchsin. Eight of the 56 strains assigned to *M. rhodochrous* in 1957 showed traces of acid-fastness. Retention of the carbol fuchsin was, therefore, accepted as a variable characteristic, providing little aid in the generic location of the species.

After more study, discussions with other investigators, and second thoughts, it is conceded that this species cannot be satisfactorily allocated to a genus until a comparative examination of the corynebacteria has also been made. Such a study will undoubtedly reveal other scientific names deserving consideration in the nomenclature of this species. In the meantime, and in the absence of a generally accepted common name, *Mycobacterium rhodochrous* is used with the understanding that it may be only a temporary designation, both generically and specifically.

There were only four strains, among the 23 of this species acquired since 1957, which initially bore generic and specific combinations of names not previously encountered. The authority for the name *Erythrobacillus havaniensis*, under which strain no. 804 was received, was not found. The specific epithet was assumed to be that of *Bacillus havaniensis* Sternberg (1892), a species previously listed in the synonymy of *Mycobacterium rhodochrous*. Jensen (1932) assigned *M. opacum* den Dooren de Jong (1927) to the genus *Proactinomyces*. As strains nos. 765 A and 765 B conformed to the original description of *M. opacum*, *Proactinomyces opaca* was accepted in the synonymy of *M. rhodochrous*. Authority for the name *Proactinomyces caprae* was not found, and this specific epithet may or may not be that of *Streptothrix caprae* Silberschmidt (1899), a species in the synonymy of *Nocardia asteroides* (Gordon & Mihm, 1957).

The cellular morphology of the 23 more recently examined strains of this species varied from coccoid forms to short branching filaments and easily accounted for several different generic designations given this species. Some cultures on glycerol agar exhibited only coccoid forms; others, pleomorphic cells; others, rods of various lengths; and others, pleomorphic rods and branching, swollen filaments. Four of the 23 strains retained traces of carbol fuchsin. The colonies of each of 20 of the 23 strains belonged to two or more of the following types: dense colonies with smooth edges or a thin halo of cells; dense colonies with a fringe or outcroppings of filaments; loosely filamentous colonies; and thin, filmy colonies of cells, sometimes resembling a medusa-head. The filaments of these 20 strains fragmented into shorter forms. Plate 2, figs. 5, 6, illustrate the colonies predominantly formed by a smooth strain of *Mycobacterium rhodochrous* and Pl. 2, figs. 7, 8, show the colonies of the same strain growing in the rough stage. The colonies of the three remaining strains and those of two of the 56 strains previously studied, were more like those of some strains of *Nocardia* spp. Although these five strains had all the other properties of *M. rhodochrous*, they could not be assigned to the genus *Mycobacterium* or to the genus *Nocardia* on the basis of their colonial morphology.

The results of the physiological tests applied to the 79 strains assigned to *Mycobacterium rhodochrous* are given in Table 5. The distinguishing characters of the species, in addition to scant, if any, retention of the carbol fuchsin, were: growth at 10°; formation of acid from sorbitol; failure to produce acid from arabinose, dulcitol, or erythritol; inability to use mucate or oxalate; and lack of growth on malachite green, methyl violet, pyronin, or MacConkey agar. Although all strains did not dissolve the crystals of tyrosine or utilize benzoate, these two reactions, when positive, aided greatly in the recognition of strains of *M. rhodochrous*. Other characteristics in the specific pattern were: failure to decompose casein; hydrolysis of starch; acid from glucose, mannitol, mannose, and trehalose; failure to form acid from lactose, α-methyl-D-glucoside, or raffinose; and use of lactate, malate, and succinate. Deviation by a strain in one or two respects from the pattern of these twenty-five more reliable characteristics did not prohibit its identification as a strain of this species.

DISCUSSION

The search for correlating characteristics common to old type strains, variants of the same strain, and new isolates of a species is never-ending in this taxonomic laboratory. The reliability of each character for the identification of a species is determined by application of the test or observation to as many strains of the particular species as possible. If 99 of 100 strains of *Mycobacterium smegmatis*, for example, use oxalate as a source of carbon, while only 40 grow on MacConkey agar, much more dependence is placed on utilization of oxalate than on growth on MacConkey agar in the recognition of strains of *M. smegmatis*. To accommodate for microbial variation, use of oxalate is supported by other similarly tested criteria such as acid-fastness; growth at 45°; lack of growth at 52°; acid formation from arabinose, dulcitol, inositol, and rhamnose; production of urease; and growth on pyronin agar. With a distinctive group of 20 to 25 reliable properties for the description of a species, variation by a strain from the pattern of reactions in one or two respects is expected, but does not interfere with the identification of the strain.

So many observations for the recognition of a strain are not feasible, of course, in a diagnostic laboratory. The use of MacConkey and oxalate or mucate agars may, however, be valuable as a presumptive test for the identification of *Mycobacterium fortuitum*. Strains of mycobacteria growing on MacConkey agar and changing the colour of the indicator, but not utilizing oxalate or mucate, can be expected, on the basis of this study, to belong to *M. fortuitum*.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Colonies of strain 448 of *Mycobacterium fortuitum* on Bennett's agar at 6 days (×680).

Fig. 2. Colony of strain 1025 of *M. fortuitum* on soil-extract agar at 10 days (×780).

Fig. 3. Colony of strain 964 of *M. fortuitum* on soil-extract agar at 12 days (×780).

Fig. 4. Portion of colony of strain 1026 of *M. fortuitum* on soil-extract agar at 10 days (×780).

Plate 2

Figs. 5, 6. Colonies of R. S. Breed's strain KBMC (W 3406) of *Mycobacterium rhodochrous* on Bennett's agar at 6 days (×560 and ×690 respectively).

Fig. 7. Colony of R. S. Breed's strain KBMC (370) of *M. rhodochrous* on Bennett's agar at 5 days (×680).

Fig. 8. Portion of colony of R. S. Breed's strain KBMC (370) of *M. rhodochrous* on Bennett's agar at 11 days (×680).

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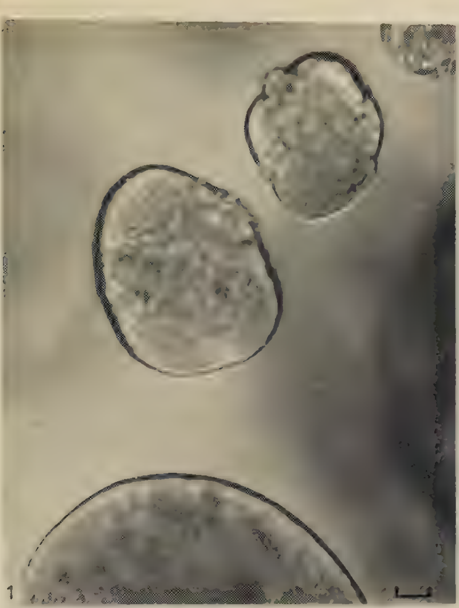

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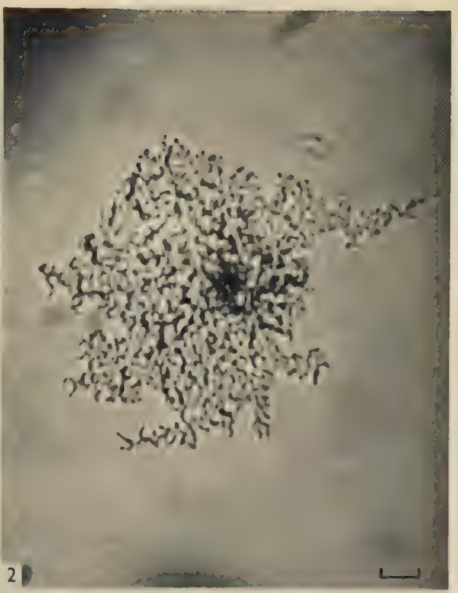

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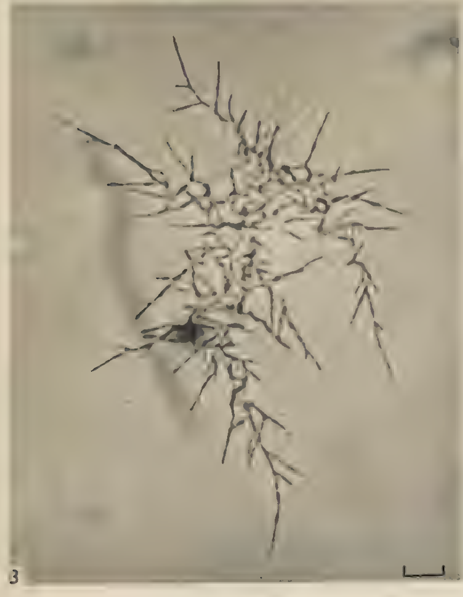

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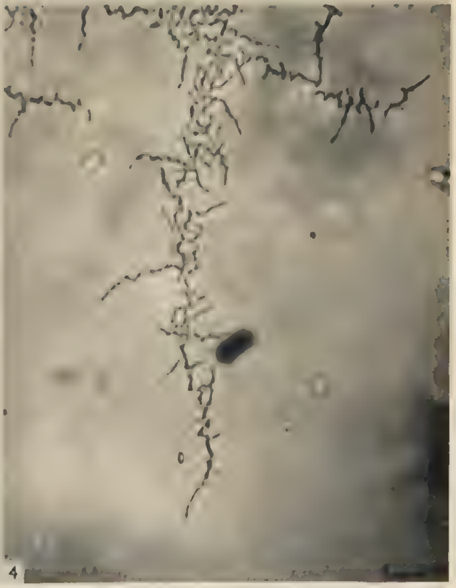

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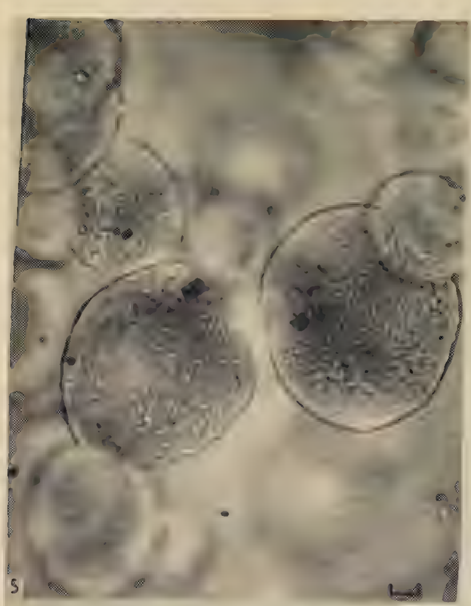
R. E. GORDON & J. M. MIHM—COMPARISON OF *MYCOBACTERIUM* SPP. PLATE 1

(*Facing p.* 748)



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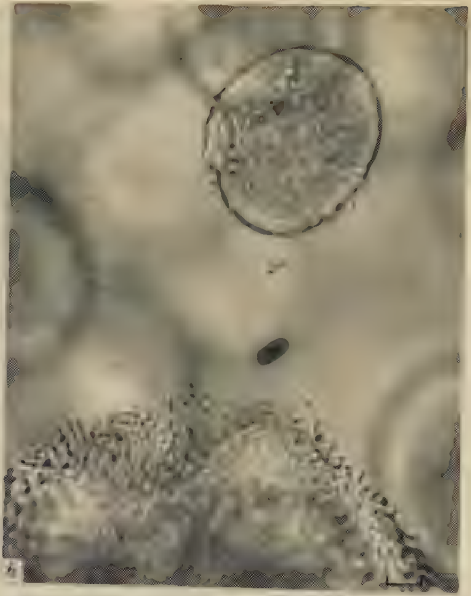

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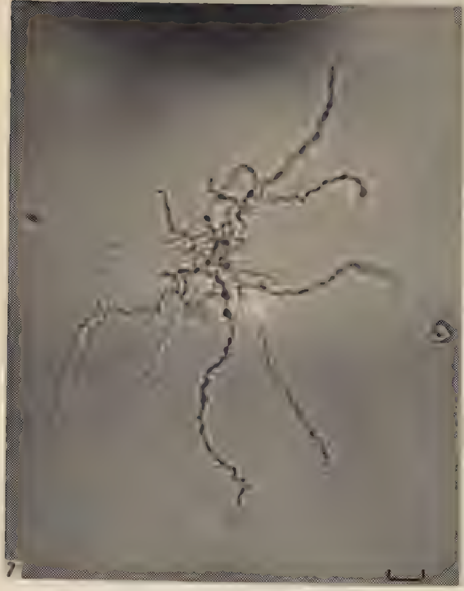

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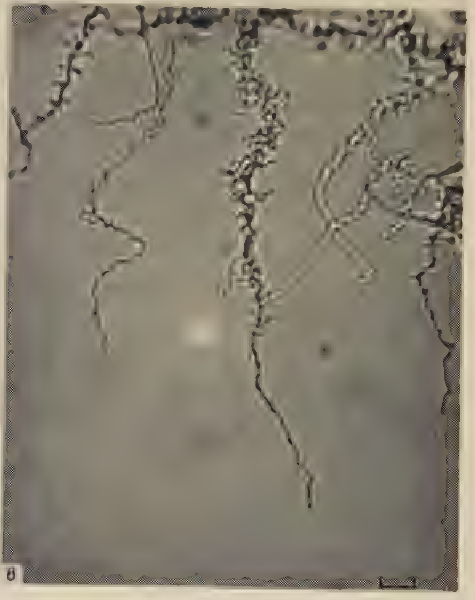

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R. E. Gordon & J. M. Mihm—Comparison of *Mycobacterium* spp. Plate 2

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